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THE IMMUNOLOGICAL BEHAVIOUR OF FRACTIONS OF SERUM GLOBULIN.

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STUDIES of serum with the ultra-centrifuge indicate that inter-molecular, and possibly intra-molecular rearrangements of globulin may be produced without drastic treatment. They suggest the possibility that the fractions prepared from serum globulin by variations of salt concentration may be artefacts, which do not exist, as such, in serum. Of the various immunological investigations of this question that have been made only two need be mentioned here. Harris and Eagle (1935), working with horse and human sera, concluded that " euglobulin " (that is, the water insoluble part of the fraction precipitated by 1/3 saturation with ammonium sulphate) and " pseudoglobulin " (the water soluble part of the fraction precipitated by 1/2, but not by 1/3 saturation with ammonium sulphate) are antigenically distinct proteins and that both are present in the whole serum. Kendall (1937) found that the " water soluble euglobulin " fraction (that precipitated by 1/3 saturation with ammonium sulphate but soluble in water) of human serum behaved like a pure protein, containing only one species of antigenic molecules. He also considered that this protein occurred in serum as such.

We have studied the question whether unfractionated serum globulin behaved as if it were a mixture of the euglobulin (water insoluble) and pseudo-globulin (water soluble) fractions.

METHODS.

Whole serum globulin was prepared with the minimum of manipulation. Horse-serum was diluted with two volumes of water and three volumes of saturated ammonium sulphate solution were added. After two hours the precipitate was centrifuged off, washed with 1/2 saturated ammonium sulphate solution and dissolved in 1 per cent. sodium chloride solution. The ammonium sulphate was removed by dialysis against 1 per cent. sodium chloride solution in the cold. This globulin solution was then passed through a Seitz filter and kept in the refrigerator.

The euglobulin and pseudoglobulin were prepared by Dr. Ogsdon, in the Laboratory of the London Hospital Medical Unit, as described by Holiday

(1936); the " euglobulin " being the fraction that separated out on dialysing whole globulin, and redissolved in 0·9 per cent. sodium chloride solution; the " pseudoglobulin " the fraction soluble in distilled water.

Rabbits were immunized by intraperitoneal injections of whole globulin, and of horse-serum that had been passed through a Seitz filter and kept in the refrigerator.

Approximately optimum proportions of Type III polysaccharide were added to 10 c.c. of Type III antipneumococcal horse-serum. The floccules were centrifuged off, washed three times with cold 1 per cent. sodium chloride solution, suspended in distilled water and injected into a rabbit's veins, twice weekly. The sera obtained from this rabbit are called " anti-floccule sera ".

Optimum proportions of anti-sera and protein solutions were estimated by the method of Dean and Webb (1926). The amount of precipitate formed by a protein with serum was measured by adding an appropriate amount of the protein solution to 1 ml. of serum, diluted with sufficient 1 per cent. sodium chloride solution to make the final volume up to 3 ml.; all solutions were at 0–5°. The mixtures were kept over night in the refrigerator, centrifuged and washed with cold 1 per cent. sodium chloride solution. The nitrogen was estimated by a micro-Kjeldahl method; the factor 6·4 was used to calculate protein. In earlier experiments the results of which were similar to those reported, the protein in the precipitates were estimated by the colorimetric method of Greenberg (1929).

AMOUNT OF PRECIPITATE FORMED WITH ANTISERA TO WHOLE GLOBULIN.

The maximum precipitate formed by either eu- or pseudo-globulin with serum 534.37.10 was less than half the maximum formed by whole globulin (Table I). The results of tests of the supernatant fluids indicate that euglobulin would not precipitate the antibody that was not precipitated by pseudoglobulin. This was confirmed by the experiments in which both eu- and pseudo-globulin were added to antiserum. It appears, therefore, that the whole globulin used was not, from the antigenic point of view a mixture of eu- and pseudo-globulin.

It is necessary to rule out the possibilities that the antiserum contained antibodies to crystalbumin and seroglycoid (Hewitt, 1936), as both of these were presumably present in the whole globulin samples; and that the difference between the amounts of precipitate formed by eu- and pseudo-globulin on the one hand, and that formed by whole globulin, on the other, may be due to precipitation of these antibodies.

It is possible to obtain crystalbumin almost pure, but seroglycoid is liable to be contaminated with globulin. The seroglycoid preparation used (which was given us by Dr. Hewitt) formed a precipitate with the antiserum to whole globulin. But the large amount of this preparation required for most rapid flocculation and for maximum precipitation suggest that the substance reacting with this serum formed only about 10 per cent. of the total protein in the preparation.

It is necessary to have some globulin preparation that can be regarded as a

TABLE I.—*The Amount of Precipitate formed with Antiserum* 534.37.10 *whole Globulin.*

Experiment No.	Protein added (mg. to 1 ml. of antiserum).	Mg. of protein in precipitate from 1 ml. of antiserum.	Tests of supernatant fluids with: Anti-serum.	Whole globulin.	Pseudo-globulin.	Eu-globulin.	Cryst-albumin.	Sero-glycoid "globulin free".
	Whole globulin							
1	0·67	4·6	±	+	+	±	..	..
2	1·46	6·4	±	+	..	..	..	..
3	1·94	6·6	+	±	0	0	0	..
4	2·91	6·4	+	..	..	..	..	..
5	3·9	4·3	..	..	..	..	..	..
	Pseudoglobulin VII							
6	0·57	2·05	+	..	..	..	..	..
7	1·0	2·40	++	++	0	±	0	..
8	2·0	1·42	++	..	..	..	..	..
	Pseudoglobulin IX							
9	0·53	2·40	+	+	+	+	+	±
10	0·7	2·73	+	..	∓	0	..	..
11	1·78	3·24	+	+	0	∓	..	+
12	2·66	3·15	++	+	0	∓	..	+
	Euglobulin							
13	0·20	0·87	∓	..	..	+	..	..
14	0·34	1·2	∓	..	..	∓	..	..
15	0·475	1·64	+	++	+	0	∓	..
16	1·02	1·56	++	++	+	0	∓	..
17	1·70	1·54	..	..	..	..	..	..
18	2·6	1·11	..	..	..	..	..	..
	Euglobulin + pseudoglobulin							
19	Eu. 0·41 / Pseudo. VII 0·57	1·85	..	+++	∓	∓	+	++±
20	Eu. 0·41 / Pseudo. VII 1·0	2·2	..	+++	∓	∓	±	+
21	Eu. 0·475 / Pseudo. IX 0·53	3·11	++	++	±	..	..	±
	Seroglycoid							
22	6·8	3·6	∓	..	..	..	..	..
23	10·2	4·83	∓	..	..	..	..	..
24	13·6	5·36	+	+	±	±	..	..
25	17·0	4·91	++	+	±	±	..	..
	Crystalbumin							
26	0·1	0·64 Approximately maximum precipitate	..	..	..	..	..	..
	Seroglycoid "globulin free"							
27	0·2	0·60 Approximately maximum precipitate	..	..	..	..	..	..

final appeal. We have assumed that the washed floccules formed by Type III polysaccharide with the corresponding horse antiserum contained globulin free from crystalbumin and seroglycoid ; and that the antiserum to these floccules would not precipitate seroglycoid nor crystalbumin, although it might also not precipitate all the globulin fractions. With the anti-floccule serum 158.37.6 the seroglycoid preparation formed a precipitate. But large amounts were required for most rapid flocculation and for maximum precipitation (Table III) again indicating the presence of a contamination with globulin equal to almost 10 per cent. of the total protein. The seroglycoid was therefore cleared of globulin by the addition of approximately the optimum proportion of anti-floccule serum 158.37.6. The resulting seroglycoid, free from globulin, which still formed a heavy precipitate with an anti-seroglycoid serum (supplied by Dr. Hewitt), now formed only a small precipitate with the anti-whole-globulin serum 534.37.10 (Table I). The total precipitates formed by eu- and pseudo-globulin, seroglycoid free from globulin and crystalbumin do not amount to more than two-thirds of the maximum precipitate formed by whole globulin. The behaviour of the preparations of eu- and pseudo-globulin with the antiserum 534.37.10 does not resemble the behaviour of two antigenically distinct proteins with a serum that contains antibodies to both (*e. g.* egg albumin and serum albumin, Dean, Taylor and Adair, 1935), but rather the cross reactions studied semi-quantitatively by Landsteiner and van der Scheer (1936), and quantitatively by Marrack and Carpenter (1938).

The relation between the flocculation times is also similar to that found in these cross reactions. With serum 534.37.10 diluted 1/8 the times of flocculation at optimum proportions were : whole globulin 19 minutes, pseudoglobulin 83 minutes, englobulin 72 minutes, seroglycoid 30 minutes.

THE AMOUNT OF PRECIPITATE FORMED WITH ANTISERUM TO WHOLE HORSE-SERUM.

With the strong antiserum 643.37.9 to untreated horse-serum (Table II) the difference between the amount of precipitate formed by whole globulin, and that formed by the two fractions was less than that found with antiserum to whole globulin. The antiserum appeared to contain a small amount of antibody to crystalbumin, as it formed a precipitate with a sample of crystalbumin, which formed no precipitate with the antifloccule serum and was therefore presumably practically free from globulin. The most rapid flocculation was obtained on the addition of small amounts of crystalbumin (0·145 mg. to 1 c.c. of antiserum) ; and the ratio of this amount of crystalbumin to total protein in the precipitate formed (1·3 mg.) was approximately equal to the ratio of antigen to total precipitate at the equivalent point in the reaction of crystalbumin with a genuine antiserum to crystalbumin (Heidelberger and Kendall, 1937). If this precipitate formation by crystalbumin and serum 643.37.9 were due to a small amount of globulin contaminating the crystalbumin, the amounts required for most rapid flocculation would be larger relative to the amount of precipitate formed, as was found with the seroglycoid preparation in its reaction with anti-globulin and anti-floccule serum. The

TABLE II.—*The Amount of Precipitate formed with Antiserum* 643.37.9 *to Whole Horse-Serum.*

Experiment No.	Protein added (mg. to 1 ml. of antiserum).	Mg. of protein in precipitate from 1 ml. of antiserum.	Tests of supernatant fluids with: Anti-serum.	Whole globulin.	Pseudo-globulin.	Eu-globulin.	Cryst-albumin.	Sero-glycoid "globulin free".
	White globulin							
1	1·46	11·7	+	++	..	..	..	..
2	1·94	13·5	+	+	±	..	..	..
3	3·98	17·3	++	∓	∓	∓	0	++
4	7·96	11·9	..	..	..	..	..	..
5	9·72	9·9	..	..	..	..	..	..
	Pseudoglobulin IX							
6	1·42	9·9	..	..	..	..	..	..
7	1·78	10·6	..	+++	+	..	..	..
8	2·66	13·5	+	++	±	..	..	..
9	3·54	13·6	+++	++	0	0	+	..
10	7·10	7·6	..	..	..	..	..	..
11	14·2	1·9	..	..	..	..	..	..
	Euglobulin							
12	1·02	7·1	∓	..	++	..	..	..
13	1·36	8·1	±	++	++	+	±	..
14	2·72	9·3	++	++	++	∓	±	..
15	5·44	7·9	++++	++	..	..	..	..
	Euglobulin + pseudoglobulin							
16	Eu. 2·66 / Pseudo. IX 2·72	11·5	+++	+	∓	0	..	..
17	Eu. 1·33 / Pseudo. IX 1·36	11·9	±	+	±	0	..	++
18	Eu. 0·89 / Pseudo. IX 1·36	11·64	+	+	±	0	..	++
	Crystalbumin							
19	0·29	1·6 (About maximum precipitate)	..	..	..	..	..	..
	Seroglycoid							
20	1·6	2·1 (About maximum precipitate)	..	..	..	..	..	..

whole globulin preparations formed a precipitate when relatively large amounts were added to anti-crystalbumin sera; as might be expected, therefore, they contain albumin and may give rise to antibodies to crystalbumin. The 1·3 mg. of precipitate formed by crystalbumin with the antiserum 643.37.9 accounts for part of the difference between the amounts of precipitate formed with this serum by whole globulin and by the globulin fractions.

The position with regard to seroglycoid is more dobutful. The maximum precipitate, 2·1 mg., was formed with 1·6 mg. of "globulin free" seroglycoid;

which makes a very low antibody/antigen ratio in the precipitate if all the " globulin free " seroglycoid was involved in the reaction. But the supernatant fluid from Expt. 3 (in which the maximum amount of precipitate was formed with whole globulin) rapidly formed a precipitate on the addition of " globulin free " seroglycoid, but with further whole globulin formed only a very slight precipitate on standing over night. It therefore appears that, at most, only a fraction of the 2·1 mg. of precipitate that can be formed by seroglycoid with serum 643.37.9 can be included in the 17·3 mg. of precipitate formed by whole globulin ; precipitate formed by seroglycoid will not account for the difference between the amount of precipitate formed by whole globulin and that formed by the globulin fractions and albumin.

If it is supposed that the reactions of eu- and pseudo-globulin with antibodies to whole globulin is a form of cross reaction, it must be supposed that the serum 643.37.9 was less specific than the antiglobulin sera tested, since with serum 643.37.9 the precipitates formed by the fractions were larger relative to those formed by the whole globulin. The serum 643.37.9 was a more potent serum, as shown by the larger content of antibody protein and more rapid flocculation. Flocculation with pseudo- and eu-globulin was again slower than with whole globulin ; serum diluted 1/10 flocculated with the optimum proportion of whole globulin in 10–15 minutes, with pseudo-globulin in 30 minutes and with euglobulin in 35 minutes. It is not unusual to find that the more potent sera are less specific.

THE AMOUNT OF PRECIPITATE FORMED WITH ANTISERUM TO SPECIFIC FLOCCULES FROM TYPE III SERUM.

It is remarkable that the same preparations of whole globulin and euglobulin which differ in their behaviour with antiserum to whole globulin, resemble each other in their behaviour with antiserum to the floccules (Table III). The amounts of precipitate formed, the optimum proportions and times of flocculation (45 minutes with serum diluted 1/6) were almost the same.

On the other hand, pseudoglobulin, which with the anti-whole-globulin serum resembled whole globulin more closely than did euglobulin, formed considerably less precipitate and flocculated more slowly (80 minutes). The globulin contaminating the specimen of seroglycoid behaved as pseudoglobulin.

Antibody protein from Type II floccules was recovered by the method of Heidelberger and Kendall (1936). With the anti-floccule serum 158.37.6, this preparation flocculated at the same time over a wide range of proportions. The flocculation time (about 60 minutes) was longer than that of eu- and whole globulin ; but the amounts of precipitate formed were greater.

With antiserum to whole globulin similar preparations of Type I and Type II antibody behaved as if they were unrelated proteins containing small quantities of whole globulin. These proteins are being investigated further.

DISCUSSION.

Our observations do not conflict with those of Harris and Eagle (1935) nor with those of Kendall (1937). These workers were not investigating the special point that we have under consideration ; that is, whether the fractions formed

TABLE III.—*The Amount of Precipitate formed with Anti-floccule Serum* 158.37.6.

Experiment No.	Protein added (mg. to 1 ml. of antiserum).	Mg. of protein in precipitate from 1 ml. of antiserum.	Tests of supernatant fluids with: Anti-serum 158.	Whole globulin.	Pseudo globulin.	Eu-globulin.	Anti-serum to sero-glycoid.	Anti-serum to whole globulin.
	Whole globulin							
1	0·48	1·85	∓	+	..	..	+	∓
2	0·63	2·85	+	+	..	..	..	..
3	1·26	3·65	++	+	..	..	..	..
4	2·43	3·65	..	..	..	..	..	..
5	3·9	3·65	++	±	..	..	..	..
6	7·8	2·25	..	..	..	..	..	..
	Pseudoglobulin VII							
7	0·35	1·35	∓	..	±	..	..	..
8	0·50	1·67	±	..	±	..	..	..
9	0·99	2·58	++	+	±	++	..	..
10	1·45	2·13	++	+	±	..	..	..
	Euglobulin						**Recovered Antibody II**	
11	0·56	2·67	..	..	..	++	..	..
12	1·12	3·66	++	++	..	+	+	..
13	2·24	3·5	++	+	∓	..	+	..
14	4·48	3·9	..	+	∓	..	+	..
15	8·98	2·7	..	..	..	..	..	..
	Recovered Antibody II							
16	0·33	1·82	∓	..	..	..	±	..
17	0·67	2·58	∓	..	..	..	±	..
18	1·34	4·33	+	..	..	..	±	..
19	2·68	5·10	+	..	..	..	∓	..
20	6·7	6·10	..	..	..	..	..	..
	Seroglycoid				**Seroglycoid (not globulin free)**		**Antiserum to seroglycoid**	
21	1·2	0·83	..	..	..	..	..	..
22	3·4	1·28	..	..	∓	..	++	∓
23	6·8	1·85	∓	++	∓	..	..	±
24	13·6	2·76	+	+	∓	..	..	+
25	27·2	2·22	+++	+	∓	..	..	..

from serum globulin will precipitate all the antibody to whole serum globulin. We did not find our pseudoglobulin and euglobulin to be immunologically distinct, as were those of Harris and Eagle ; but we did not go so far in the process of separating (or making) the two fractions. We only separated whole globulin in water insoluble (euglobulin) and water soluble (pseudoglobulin) ; whereas the immunologically distinct proteins of Harris and Eagle were the water insoluble part of the globulin precipitated by 1/3 saturation with ammonium sulphate, and the water soluble part of the fraction precipitated by 1/2 saturation, but not by 1/3 saturation. According to Kendall's view

both our pseudoglobulin and our euglobulin contained a-globulin and X-globulin. But, with anti-floccule serum euglobulin precipitated as much antibody as whole globulin, while pseudoglobulin precipitated considerably less, however much was added. It is difficult to account for these reactions on any simple theory of mixtures or cross reactions.

Ando and his colleagues (1937) have shown that fractions, which differ antigenically, are present in the globulin of horse-serum. This is the counterpart of the differences found between globulin molecules in their antibody functions. It does not follow, however, that these immunologically different fractions correspond to fractions into which globulin is separated by variations of salt concentration.

The behaviour of the antibody, recovered by the method of Heidelberger and Kendall (1937), both with the anti-floccule serum and with antiserum to whole globulin is remarkable and is being investigated further.

SUMMARY.

The behaviour of the water soluble and insoluble fractions of serum globulin with antiserum to whole serum globulin has been investigated quantitatively. The results suggest that these fractions are not present, as such, in the whole globulin.

Our thanks are due to Dr. Hewitt for supplies of seroglycoid and anti-seroglycoid serum.

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THE TRANSMISSION OF INFLUENZA VIRUS "W.S." TO THE CHINESE MINK AND THE DAVID'S SQUIRREL.

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Up to the year 1936 ferrets, mice, swine and hedgehogs seemed to be the only animals which had been used for experimental study of epidemic influenza since the discovery of the virus (Smith, Andrewes and Laidlaw, 1933). In the present communication an account is given of attempts to transmit the infective agent to minks, young squirrels, and other animals.

The "W.S." strain of influenza virus originally isolated by the English authors was brought back from the National Institute for Medical Research, Hampstead, London, and used for the present work. For initial transmission experiments "gradocol" membrane (0·76μ) filtrates prepared from mouse passage virus were employed, in a dose of 0·5 c.c. for squirrels and 1 c.c. for minks. All inoculations were given under ether anæsthesia. The inoculated animals were kept under observation for 4–5 days for clinical signs of infection. They were then sacrificed, and their lungs and nasal turbinates examined carefully. When lesions were conspicuous the tissues were thoroughly ground up with Pyrex glass powder, and 5–10 per cent. suspensions prepared from them, for further inoculation; 5 per cent. emulsions were used whenever membrane filtrates were required. As a rule a group of 2 or 3 mice was also inoculated with such material for identification of the virus.

INFLUENZA IN CHINESE MINKS.

Chinese minks or *Mustela sibirica*, Milne-Edwards, are very abundantly found in Central and Eastern China from Yangtze Delta southward to Fukien. They frequent human dwellings as well as open country in the neighbourhood of creeks and streams (Sowerby), and they can be trapped with ease. They possess partially webbed feet, and are closely related to the ferret or polecat.

It was found that Chinese minks are highly susceptible to the influenza virus. Clinically about 24 hours after intranasal instillation there was usually a febrile response lasting for several days, during which respiration was greatly embarrassed and was of the abdominal type. The eyes were generally watery, but no other sign of coryza such as sneezing or excessive nasal secretion was observed.

Serial transmission.—As will be seen from Table I, the virus has been transmitted from mink to mink without any difficulty, and judging from the progressive character and extensiveness of pulmonary lesions it seems that serial passages may be carried on indefinitely, but on account of the sudden outbreak of war in Shanghai only 9 passages could be made.

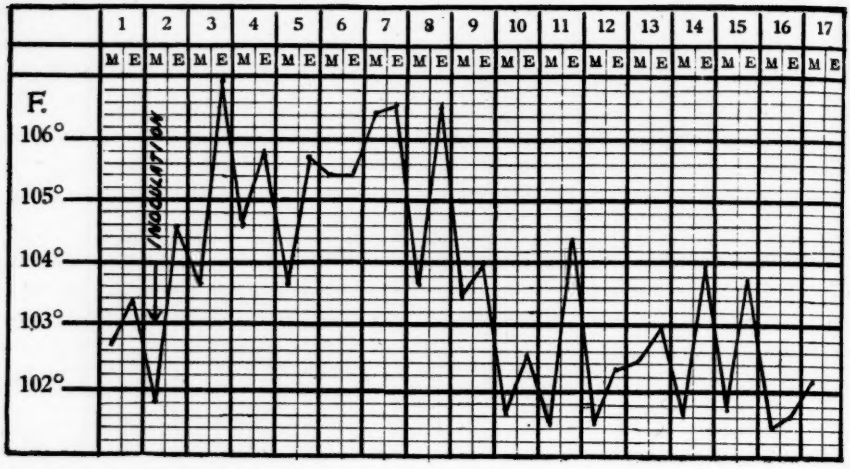

CHART I.—Temperature variations in Mink No. 8 infected with the virus of influenza.

TABLE I.—*Serial Transmission of Influenza Virus in Chinese Minks.*

Mink No.	Passage No.	Inoculation material.	Autopsy findings; extent of pneumonic consolidation.	* Lesions in mice inoculated with emulsion of—	
				Lungs.	Turbinates.
2	1	Stock virus	Azygos complete, and dorsal side of right lower lobe	..	..
3	2	Lung suspension, Mink 2	Complete in right mid-lobe; 1 × 1 cm. left upper lobe; 1/3 azygos and 1 × 2 mm. scattered over all lobes	32	33
4	3	Lung filtrate, Mink 3	2 × 1 mm. right upper lobe at base	(Lung and turbinates) 33	
5	4	Lung and turbinate suspension of Mink No. 4	5 × 10 mm. left upper; 5 × 5 mm. right upper; 1 × 2 mm. well scattered	33	33
6	5	Lung suspension, Mink 5	2 × 4 mm. left lower lobe; other lobes all complete	44	44
7	6	Lung suspension, Mink 6	Azygos complete; 1/2 left upper and lower; right lower nearly complete	34	24
8	7	Lung filtrate, Mink 7	Complete hepatization of all lobes	34	42
9	8	Lung filtrate, Mink 8	Died 40 days after inoculation. Extensive areas of consolidation	000	..

* In this and other tables each figure in the two final columns represents a mouse. The number indicates the size of lung lesions. 1 denotes lesions of 1 mm. in size, while 4 means complete or nearly complete hepatization; 2 and 3 intermediate.

Besides its presence in the lungs and nasal turbinates post-mortem, the virus has also been found in the naso-oral washings of one mink (No. 9)

obtained on the 6th day after inoculation. Similar material taken on the 10th day was not infectious.

The intensity of pathological changes in minks was found to increase with the number of transfers. There was only a small patch of pneumonia in the 1st generation, while by the 7th transfer (with filtered material) the whole of the lungs were completely hepatized. Mink No. 9, which died 41 days after the inoculation, still showed what appeared to be extensive areas of pneumonia, but on account of the war no sections of the tissues were possible. A retarded process of resolution and repair has been observed in mice recovering from influenzal infection, and delayed resolution may perhaps be a characteristic of influenzal pneumonia.

No opportunity has yet arisen to test the possibility of direct transmission of influenza virus to minks from human sources. Should this be proved possible, then minks would provide a satisfactory substitute for ferrets in the study of epidemic influenza in places where the latter animals are not available.

INFLUENZA IN SQUIRRELS.

Three species of squirrels have been tested, namely David's squirrels (*Sciurotamias davidianus*, Milne-Edwards), striped ground squirrels or chipmunks (*Eutamias asiaticus senescens*, Miller) and Chekiang squirrels (*Callosciurus caniceps canigenus*, Howell).

The preliminary tests appeared to indicate that the Chipmunk and the Chekiang squirrel were resistant, whereas the David's squirrel was susceptible to infection with the " W.S." strain of influenza virus. The susceptibility of this species was further tested by instillation of diluted virus intranasally, when it was found that a 1 in 10,000 dilution of a filtrate from a 5 per cent. emulsion of infected mouse lung usually produced what appeared to be small, typical lung lesions.

Working with *Spirochæta recurrentis*, Meleny (1927) found that splenectomized squirrels (*Sciurus vulgaris*) were more susceptible to the organism than normal animals of the same species. No such difference between splenectomized and normal David squirrels could be demonstrated in case of the influenza virus.

Attempts to infect David squirrels by subcutaneous, intraperitoneal, intravenous and intracranial routes have not been successful, and transmission by contact infection was also negative.

Transmission by serial passage.—The results of serial passage experiments are given in Table II.

It is of interest to note that although serial passage of the virus was easily effected in the beginning, after 6 or 7 generations the virus suddenly tended to die out. As shown in Table II we have, however, succeeded in carrying the virus to the 10th generation, keeping it alive in squirrel tissues for 48 days. Further transfers have not been attempted.

Lung tissues of normal squirrels are apt to look pneumonic when left at room temperature during hot weather, probably due to rapid setting in of atelectasis. This fact should be borne in mind and control measures taken when these animals are used for influenza study.

TABLE II.—*Serial Passage of Influenza Virus in David's Squirrels.*

Squirrel No.	Passage No.	Inoculation material.	Autopsy findings; extent of pneumonic consolidation.	Lesions in mice inoculated with emulsion of—	
				Lungs.	Turbinates.
58	1	Stock virus filtrate	3 × 4 mm. left lobes	001	000
64	2	*Lung, Squirrel No. 58	3 × 4 mm. azygos lobe	321	110
80	3	,, ,, ,, 64	1 × 1 cm. left lower lobe	433	024
91	4	,, ,, ,, 80	3 × 4 mm. left upper and lower lobes. Complete, right mid-lobe	30	30
93	5	,, ,, ,, 91	10 × 10 mm. left upper and lower lobes	32	31
95	6	,, ,, ,, 93	10 × 10 mm. left lower lobe	32	22
101	7	,, ,, ,, 95	Negative	00	11
102	7	Turbinates ,, ,, 95	1 × 1 cm. right mid-lobe; 3 × 3 mm. right lower	4?	1?
103	8	Lungs ,, ,, 102	Negative	2?	..
105	8	Lung filtrate, Squirrel No. 102	3 × 4 mm. left lobes	20	32
106	9	Lung filtrate, Squirrel No. 105	Negative	13	00
127	9	Lung suspension, Squirrel No. 105	3 × 10 mm. left upper lobe	21	..
129	10	Lung suspension, Squirrel No. 127	3 × 3 mm. left upper lobe	10	21

* Supernatant fluid of lung suspension.

The results of serial passage of influenza virus in Chinese minks and David's squirrels form an interesting contrast. There can be no doubt that the former animal is much more susceptible.

Serological Changes.

It has been demonstrated (Smith, Andrewes and Laidlaw, 1933) that recovery from influenza in ferrets, as also in man, is accompanied by the development of serum antibodies. Such have also been demonstrated in hedgehogs (Stuart-Harris, 1936). In order to ascertain whether squirrels and minks developed immune bodies on recovery from infection, neutralization experiments have been made with their sera. The technique was the same as that described by Laidlaw, Smith, Andrewes and Dunkin (1935), and the results are given in Table III.

It will be observed that antibodies were absent in the squirrels before inoculation, and their development in convalescence is strong confirmatory evidence of their susceptibility to infection with virus, and gives a complete analogy with the disease as found in ferrets, hedgehogs and minks.

Attempts were made to transmit influenza infection to other species, including guinea-pigs, white rats, rabbits and young chicks. In no instance was successful transmission demonstrated. Stuart-Harris (1937) has shown that influenza virus may be propagated as an inapparent infection in both rats and guinea-pigs, but it is clear from his results that these species are not sufficiently susceptible to be of much use in the experimental study of the

TABLE III.—*Serum Neutralization.*

Sera.	Serum dilutions. 1/2.	1/10.	1/50.	1/200.
Immune horse-serum IH_2	..	00	00	00
Normal horse-serum	..	23	22	33
Squirrel No. 97, before inoculation	22	?1	23	..
7 days after	00	01	01	..
14 ,, ,,	00	00	?0	..
Squirrel No. 98, before inoculation	33	33	33	..
7 days after	00	00	11	..
Mink No. 9, 15 days after inoculation	002	001	001	..
34 ,, ,, ,,	001	000	344	..

disease. McIntosh and Selbie (1937) describe infection of rabbits and guinea-pigs with a strain of virus which they isolated from the 1936 London epidemic, but in our hands the " W.S." strain of influenza virus did not appear to infect either of these species.

CONCLUSIONS.

1. The Chinese mink (*Mustela sibirica*) is fully susceptible to the " W.S." strain of influenza virus, and it is suggested that this species might be used instead of the ferret for influenza studies.

2. David's squirrel (*Sciurotamias davidianus*, Milne-Edwards) is also susceptible to influenza infection, but the virus may die out in serial transmission. Nevertheless, serial passage was maintained for ten generations.

3. Chipmunks (*Eutasias asiaticus senescens*, Miller), Chekiang squirrels (*Callosciurus caniceps canigenus*, Howell), rabbits, white rats, guinea-pigs and young chicks did not prove to be susceptible to the " W.S." strain of influenza virus.

The writer is indebted to Sir Patrick P. Laidlaw, Dr. C. H. Andrewes and Dr. Wilson Smith, of the National Institute for Medical Research, Hampstead, London, for the supply of the immune horse-serum IH_2 and the " W.S." strain of influenza virus. His thanks are also due to Mr. Arthur de C. Sowerby for the identification of the squirrels and minks, and to Sir Patrick Laidlaw for advice in the presentation of results.

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THE ENZYMIC ACTIVITY OF VACCINIAL ELEMENTARY BODIES.

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IN discussions concerning the nature of viruses the statement is often made that viruses possess no independent metabolism. Actually there is little evidence either for or against this statement. Experimental work on this point has been confined to testing a few viruses for a few enzymes, and the fact that the results have been mainly negative is no proof that viruses in general are devoid of any metabolic activity.

Since viruses can at present be grown only in the presence of living cells, there are only two methods available for studying their metabolism. The first consists in comparing the metabolic activity of virus-infected tissue with that of similar uninfected tissue. It is clear that ignorance of the relative amounts of virus and tissue present, and of the effect of infection *per se* on the normal metabolism of the tissue will make the interpretation of the results exceedingly difficult. This method was adopted by Bronfenbrenner and Reichert (1926) working with the viruses of herpes simplex and rabies, and by Holmes (1937) with vaccinia virus ; in both cases it was found impossible to attribute any O_2 uptake to the virus examined. Kempner (1927), using the same principle, found a marked difference in the O_2 uptake of plasma from hens infected with fowl plague compared with that of normal controls ; the respiratory mechanism involved could also be differentiated from the normal, since it was not inhibited by octyl alcohol. He pointed out that this respiration might be ascribed either to the virus, or to the effect of the virus on the normal plasma.

The second method depends on separating the virus from the tissues of the host, so that its independent metabolism, if any, can be studied. In this case two criteria of a successful technique must be satisfied. Firstly, in order to assess the significance of negative results a quantity of the virus comparable on a weight basis with other enzymic material must be available for testing ; and secondly, the purity of the material used must be such that any positive results can be ascribed to the activity of the virus alone. The only viruses which have been obtained in a form which satisfies both these criteria unequivocally are those isolated as crystalline, or paracrystalline, heavy proteins, *e. g.* tobacco mosaic virus. As yet there is no information as to their metabolic activity.

It has been possible, however, to obtain other viruses in a relatively high state of purity, and in weighable amounts. Schüler (1935) examined the metabolic activity of a bacteriophage prepared according to the method of Schlesinger (1933). He found no detectable O_2 uptake in the presence of

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glucose or lactate with quantities of phage equivalent to 17 mg. dry weight; quantities equivalent to 1 mg. dry weight were tested for the presence of other enzymes, *e. g.* trypsin, papain, maltase, catalase, urease, etc., but only phosphatase was recognized. Craigie (1932) described a method for preparing suspensions of vaccinial elementary bodies by which large amounts of elementary bodies in a high state of purity could be obtained. Using such material Parker and Smythe (1937) showed that vaccinial elementary bodies did not utilize glucose or hexosemonophosphate either aërobically or anaërobically.

It appears, therefore, that the only substances which have been tested as substrates for the respiration of these two agents are glucose, hexosemonophosphate, and lactate. While the findings of Schüler and of Parker and Smythe certainly indicate the absence of enzymes activating the particular substrates tested, the presence of enzymes catalysing other energy-producing reactions is not thereby excluded. One may instance as a possible parallel the case of the anaërobe, *Cl. sporogenes*, which exhibits no dehydrogenase activity towards glucose, lactate, or succinate, but can obtain energy sufficient for growth by other reactions (Stickland, 1934, 1935; Fildes and Richardson, 1935).

Craigie's method for the preparation of vaccinial elementary bodies from rabbit-skin pulp has been employed by one of us for the past two years to obtain material for serological investigations, and certain minor modifications have been described in a previous paper (Salaman, 1937). In its present form this method provides a means of obtaining simply and economically very large quantities of elementary bodies, free from all but negligible amounts of cellular detritus or of soluble impurities. This has made feasible a more extended study of the enzymic activity of washed vaccinial elementary bodies, the preliminary results of which are now reported.

TECHNIQUE.

The strain of vaccinia used has been previously described (Salaman, 1934, 1937) and will be designated the " B strain ". It was originally derived from sheep-lymph (Lister Institute No. 1715). Before sheep-lymph, which is usually propagated sheep—rabbit—sheep, will give a satisfactory yield of elementary bodies, it must be subjected to a number of serial passages in the same species of host. The B strain had been passed more than 70 times on rabbits' skin at the beginning of this work.

2 ml. of a suspension of elementary bodies in 50 per cent. glycerin are rubbed into the freshly shaved skin of a rabbit's back with a rubber glove. Four days later the rabbit is killed; the skin is removed and pinned out on a board, swabbed twice with pure ether and then wetted thoroughly with a buffer solution (M/250 citric acid-disodium phosphate, pH 7·1). After a few minutes the skin is vigorously scraped with a sharp bone scoop. The scrapings are placed in a large centrifuge tube and more buffer solution added, to a total volume of 25 ml. (per rabbit). The tube is shaken mechanically for $\frac{1}{2}$ hour, and spun at 5000 r.p.m. for 5 minutes. The supernatant fluid is removed, and the deposit resuspended in another 25 ml. of buffer solution and the process repeated. A third extraction is sometimes added. The pooled

supernatant fluids are spun on a Lundgren angle centrifuge in ordinary $6 \times \frac{3}{4}$ inch hard glass test-tubes, holding 20 ml. each, for $1\frac{1}{2}$ hours; this deposits the elementary bodies as well as the larger cellular detritus, leaving lighter or smaller particles in the supernatant fluid (1st supernatant, Table IV). The deposit is resuspended in 5 ml. of buffer solution and spun on an ordinary centrifuge at 3000 r.p.m. for $\frac{1}{2}$ hour. The supernatant fluid is removed and saved, and the deposit resuspended in another 5 ml. of buffer solution and the process repeated; this is done four times in all, and the supernatant fluids pooled. This process gets rid of practically all the remaining cellular detritus, and avoids the great loss of elementary bodies which is involved when the preparation is given only one spinning in bulk and the deposit discarded. The pooled supernatant suspensions of elementary bodies are spun again, on the angle centrifuge, and the deposits washed and redeposited several times (see Table IV), and finally suspended in buffer solution (about 10 ml. per rabbit), or in other fluids in special cases.

The final preparations, which will be referred to as elementary body (E.B.) suspensions, have the appearance of milk, and remain stable for several weeks (in sharp contrast to preparations which contain an appreciable amount of tissue detritus, which rapidly form deposits on standing). They contain, as a rule, from 20 to 30 mg. of dry matter per rabbit, but as much as 60 mg. have been obtained. Their viable bacterial content, tested aërobically, is low, varying from 100 to 300 organisms per ml. in most cases. Their infective titres, as judged by the intracutaneous injection of 0·2 ml. of serial tenfold dilutions into the rabbit, vary between 1 in 10^6 and 1 in 10^8. On microscopical examination, either by dark-ground illumination in the fresh state, or by transmitted light when fixed and stained, they appear to consist almost exclusively of the small round bodies of uniform size, shape, and refractility which are characteristic of this virus. Dr. A. S. McFarlane has kindly examined the sedimentation of a number of these suspensions by the ultra-violet absorption technique, and finds that their behaviour indicates a degree of homogeneity comparable with that of preparations of the heavy protein of tobacco-mosaic virus (McFarlane, 1938).

Nitrogen (Kjeldahl) estimations were made on four different suspensions, and values from 13·0 to 13·3 per cent. N (calculated on the dry weight of the bodies) were obtained. These results agree with those of Hughes, Parker, and Rivers (1935). Two suspensions of bodies, which had been washed repeatedly in M/1000 NaCl or in distilled water till free of phosphate buffer, were found to contain 0·75 and 0·73 per cent. P respectively (calculated on the dry weight of the bodies).

Consideration of all these properties, which have been remarkably constant in a large number of preparations, leaves little doubt that the proportion of foreign matter present can only be extremely small.

ENZYMIC ACTIVITY.

Dehydrogenase activity was tested by the usual Thunberg technique, at pH 7·2 and 37°.

Preliminary experiments showed that methylene blue even in very small concentration (0·2 ml. M/20,000) was not reduced by the E.B. suspensions in

the presence of a variety of substrates. Methylene blue, however, is known to have an inhibitory effect on dehydrogenase activity (Quastel and Wheatley, 1931) and it has been shown that certain viruses, including vaccinia, are highly sensitive to the photodynamic action of methylene blue in presence of oxygen (Perdrau and Todd, 1933). In most subsequent experiments, therefore, methylene blue was replaced by 2 : 6 dichlorophenolindophenol, the more positive oxidation-reduction potential of this dye giving the further advantage that a possibly feeble reducing intensity might be more readily demonstrable. Other dyes of the indophenol series were tested also, without affecting the results.

Keilin-Thunberg tubes were used, the dye being placed in the hollow stopper and mixed after evacuation. The test mixtures were as follows : 0·5 ml. E.B. suspension (dry weight 1·0 to 1·5 mg.) ; 0·5 ml. 0·1 *M* substrate ; 0·2 ml. M/2000 2 : 6 dichlorophenolindophenol or M/10,000 methylene blue. The dehydrogenase activity of the virus was negligible (no reduction in 4 hours) towards all the substances tested, viz. glucose, hexosemonophosphate, *a*-glycerophosphate, succinate, lactate, acetate, formate, *dl*-malate, glycine, alanine, leucine, and peptone.

Only one preparation (B 80) gave positive results, towards succinate (10 minutes), hexosemonophosphate, and *a*-glycerophosphate (50 minutes). This particular preparation was found microscopically to be less homogeneous than the others, and it is probable that the activity in this case was due to accompanying tissue detritus.

The dehydrogenase activity of well-washed suspensions of *Bact. coli*, baker's yeast, and finely divided liver-tissue was tested under identical conditions, the experiments serving as positive controls for the technique and for comparison of the enzymic activity on a dry weight basis (determined throughout on aliquot samples). The reduction times for quantities from 1·0 to 1·5 mg. dry weight were approximately 5 to 10 minutes in presence of succinate or lactate.

Phosphatase activity.—Seven different preparations were tested for phosphatase, with positive results in every case. Typical protocols are given in Table I. The phosphatase activity was higher at an alkaline pH than at pH 6·8 and was increased by the addition of magnesium.

The values for A/W (= mg. P hydrolyzed in 1 hour at 37°/mg. dry weight enzyme) obtained for different preparations in presence of magnesium were approximately 0·005 at pH 6·8 and from 0·03 to 0·05 at pH 9·0 ; one preparation (B 78) had A/W = 0·11 (pH 9·4) ; those for the detritus were slightly lower. Tested under similar conditions a sample of purified bone phosphatase gave A/W = 0·65 (pH 9·0) and a sample of *Bact. coli* gave A/W = 0·02 (pH 8·0). The phosphatase activity of the elementary body suspensions is therefore of the same order as that of representative tissues and micro-organisms tested under similar conditions.

Catalase activity.—Five different preparations were tested for catalase with positive results in each case. The experiments shown in Table II were primarily qualitative. The velocity constant k of H_2O_2 decomposition under standard conditions at O° and pH 6·8 was measured for one preparation (B 84, Table III) from which the value *Kat. f.* (= k/g. enzyme) = 6·4 was

TABLE I.—*Phosphatase Activity.*

Method.—The activity was tested on Na β-glycerophosphate (final concentration ·066 *M*) ∓ $MgSO_4$ (·003 *M*). At pH 9·0, or above, veronal buffer was added (final concentration 0·02 *M*). The test mixtures contained 0·5 ml. enzyme (suspended in phosphate citrate buffer) and were made up with H_2O to a total volume 1·5 ml. After incubation for 1 hour at 37° at the stated pH, 0·5 ml. 10 per cent. trichloroacetic acid was added, and the inorganic phosphate in the supernatant estimated colorimetrically.

pH.	Enzyme preparation.	Mg. dry weight (=W).	Activity (=A) mg. P hydrolyzed.		A.W.	
			Without Mg.	+ Mg.	Without Mg.	+ Mg.
6·8	E.B. suspension B 80	1·04	·004	·005	·004	·005
	,, ,, B 84	1·42	..	·008	..	·006
	,, ,, B 87	1·95	..	·006	..	·003
8·4	,, ,, B 80	1·04	·011	·025	·011	·024
	,, ,, B 81	1·02	..	·031	..	·030
	,, ,, B 82	1·0	..	·017	..	·017
9·0	,, ,, B 84	1·42	..	·075	..	·053
	,, ,, B 87	1·95	..	·062	..	·032
	,, ,, B 87	0·98	..	·029	..	·030
9·4	,, ,, B 78	1·50	·036	·160	·024	·107
6·8	Detritus 87	9·90	..	·056	..	·006
8·4	,, 81	1·25	..	·014	..	·011
9·0	,, 87	0·40	..	·016	..	·040
	,, 87	9·90	..	·204	..	·020
6·2	*Bact. coli*	1·32	·032	·040	·024	·030
7·2	,, ,,	1·32	·027	·023	·020	·018
8·0	,, ,,	1·32	·022	·026	·017	·020
9·0	Bone phosphatase (purified)	0·25	..	·162	..	·648

TABLE II.—*Catalase Activity at* 37°.

Method.—1·0 ml. H_2O_2 + 1·0 ml. E.B. suspension in phosphate-citrate buffer pH 7·2. Incubated at 37°; H_2O_2 estimated after addition of 5 ml. 10 *N* H_2SO_4 and 4 ml. 5 per cent. KI by titration with ·005 *N* thiosulphate.

E.B. suspension.	Dry weight (mg.).	Incubation time (min.).	H_2O_2 molarity.	Titre ml. thiosulphate.
(1) None	..	0	·02	3·98
,,	..	30	·02	3·80
B 81	0·5	0	·02	3·90
,,	0·5	30	·02	0·25
,,	0·1	30	·02	2·28
Boiled	0·5	30	·02	3·71
(2) None	..	0	·04	8·05
,,	..	30	·04	8·06
B 82	0·2	30	·04	3·15
Boiled	0·2	30	·04	8·00
B 84	0·56	30	·04	1·32

TABLE III.—*Catalase Activity at* 0°.

10 ml. ·01 N H_2O_2 + 2 ml. 0·02 *M* phosphate buffer pH 6·8 + 0·5 ml. E.B. suspension. 5 ml. 10 *N* H_2SO_4 and 4 ml. 5 per cent. KI added; titration with ·01 *N* thiosulphate.

E.B. suspension dry weight.	Time.	Titre (ml.).	Velocity constant (*k*).	*Kat. f.* (=k/g enzyme).
0	0	9·86	..	..
0	30 min.	9·84	..	..
B 84 0·28 mg.	10 sec.	9·82	..	..
..	5 min.	9·61	·002	7
..	10 ,,	9·37	·002	
B 84 0·56 mg.	10 sec.	9·75	..	..
..	5 min.	9·35	·0036	5·7
..	20 ,,	8·51	·0029	

TABLE IV.—*Distribution of Enzymic Activity between Elementary Bodies, Successive Washing Fluids, and Tissue Detritus.*

Phosphatase activity: mg. P hydrolysed in 1 hour at 37°.
Catalase ,, : mg. H_2O_2 decomposed in 30 minutes at 37°, pH 7·2.

Fraction.	Volume (ml.).	Dry weight (mg.).	Total enzymic activity in fraction. Phosphatase. (pH 8.4).		Catalase.
				pH 9.0 / pH 6.8	
B 81 1st supernatant*	200	..	8–12		40,000
2nd ,,	50	65	0·5		298
3rd ,,	30	..	0		12
4th ,,	30	..	0		4
Washed detritus	..	75	0·8		27
,, elementary bodies	..	62	2·0		39
B 87 1st supernatant	15	..		2·6 / 1·5	15,000
2nd ,,	15	..		0·3 / 0·18	16
3rd ,,	15	..		0 / ..	0·5
4th ,,	15	..		0 / ..	0
5th ,,	15	..		0 / ..	0
Washed detritus	..	4·0		0·16 / ·04	2·2
,, elementary bodies	..	19·5		0·62 / ·06	7·3

* 1st supernatant = Supernatant fluid from 1st deposition of elementary bodies on the angle centrifuge.
2nd to 5th supernatants = Successive washings of elementary bodies, after removal of detritus. Detritus was similarly washed, using an ordinary centrifuge at 3000 r.p.m.

obtained; the comparable figure obtained for a sample of baker's yeast was 0·5.

Both phosphatase and catalase are widely distributed tissue enzymes, and it is therefore not surprising that both these enzymes should be present in the tissue fluids and detritus from which the elementary bodies are separated; in particular it was found that the catalase content of the first supernatant was extremely high, presumably owing to the presence of laked blood. The phosphatase and catalase activity of all the fractions obtained in the course of preparation of the suspensions was investigated; the distribution of enzymic activity is shown in Table III. It is clear that the enzymic activity of the elementary body suspensions is much greater than can be accounted for by the unavoidable carry-over of liquid at each successive washing, and is therefore not due to the presence of soluble tissue enzymes. The possibility that such enzymes are adsorbed on the virus particles cannot be dismissed, but if this is the case the enzymes are certainly not easily eluted.

The tissue detritus, containing some entangled virus, which is deposited by centrifuging at low speeds, has a marked succinic dehydrogenase activity, in contrast to the suspensions of elementary bodies. The phosphatase and catalase activity of the detritus, which may be due in part to its content of virus, is, however, of approximately the same order as that of the virus suspensions. If therefore the activity of the virus suspensions is to be attributed merely to the presence of tissue, the latter must constitute at least 50 per cent. of the dry matter present. This is quite inconsistent with the results of the bacteriological, physical and chemical examination described in the first section. In fact the suspensions showed such a high degree of homogeneity that the most reasonable assumption is that the enzymic activity is due to the virus.

It is possible that the negative findings in the dehydrogenase tests may be due to a peculiar instability of these enzymes, or to unsuitable conditions. It has been shown that the dehydrogenase activity of two filter-passing organisms, Agalactia and the sewage organism C, is very variable and readily disappears on standing (Pirie and Holmes, 1933; Pirie, 1937). It appears, on the whole, that the filter-passing organisms which have been grown *in vitro*, such as the organism causing bovine pleuropneumonia (Holmes and Pirie, 1932), Agalactia, and the sewage organisms A and C (Holmes, 1937; Pirie, 1937), activate far fewer substances as hydrogen donators than, for instance, *Bact. coli*; further search may yet reveal a suitable substrate for vaccinia virus. The results so far can be summed up by saying that vaccinia virus shows no dehydrogenase activity towards the substrates tested, while, under similar conditions, that of comparable quantities of tissues or bacteria is readily demonstrable. Incidentally the freedom from those dehydrogenases which are commonly found in tissues gives an indication of the purity of the preparations, and increases the probability that the phosphatase and catalase present in the suspensions are integral parts of the virus.

The presence of the latter enzyme is especially interesting in view of the suggested "peroxidative" *rôle* of this enzyme in coupled oxidations (Keilin and Hartree, 1936). A remarkable effect of added catalase on the oxidation of glucose by the sewage organism C has been reported by Pirie (1938).

It would be premature at this stage to discuss the significance of these results, and the investigation is being continued.

SUMMARY.

1. A method is described for the preparation of suspensions containing large quantities of vaccinial elementary bodies in a high state of purity. The results of bacteriological, physical, and chemical examinations of such suspensions indicate that the amount of foreign matter present is extremely small.

2. The elementary body suspensions were tested for the presence of certain enzymes, similar tests being carried out on the various fractions separated from the virus during its purification. All the elementary body suspensions contained phosphatase and catalase, but showed no dehydrogenase activity towards a number of substrates.

3. Reasons are given for considering that the phosphatase and catalase present are inherent in the virus.

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FURTHER STUDIES ON THE COMBINATION OF VACCINIA WITH ANTI-VACCINIAL SERUM: THE ACTION *IN VITRO* OF NEUTRALIZING ANTIBODY ON THE ELEMENTARY BODIES.

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THE mode of action on viruses of anti-viral sera has been the subject of much experiment and speculation. The fundamental observation, that of reduction or suppression of the infectivity of a virus in the presence of its anti-serum, was first made by Sternberg (1892, 1896), using vaccinia. Virus-neutralization, as it is usually called, by anti-serum has been described for the great majority of viruses, since Sternberg's first demonstration of the phenomenon. Those characters of the reaction which have attracted most attention, and which are strikingly similar in many different serum-virus systems, may be briefly summarized as follows:

(1) A serum-virus mixture which is non-infective when injected by one route, *e. g.* intracutaneously, may be infective when injected by another, *e. g.* intravenously, or intracerebrally (Todd, 1928; Andrewes, 1928).

(2) A non-infective serum-virus mixture may be made infective by simple dilution (Bedson, 1928; Todd, 1928; Andrewes, 1928).

(3) Infective virus may be recovered, sometimes apparently quantitatively, from neutral serum-virus mixtures by simple physical methods, such as centrifugation, filtration, adsorption, or electrophoresis (Andrewes, 1928; Long and Olitsky, 1930; Sabin, 1935).

(4) Recovery of infective virus by dilution or separation becomes increasingly difficult as contact between virus and anti-serum before injection is prolonged (Bedson, 1928; Andrewes, 1930, 1932; Craigie and Tulloch, 1931).

(5) After the removal of the virus from a neutral serum-virus mixture, or from one containing a moderate excess of virus, the serum may appear to have lost none of its virus-neutralizing power (Andrewes, 1928; Sabin, 1935). Absorption of a serum with a large excess of virus, however, reduces or entirely removes its neutralizing power (Smith, 1930; Salaman, 1937).

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Certain of these phenomena have analogues in phage-antiphage systems. Burnet, Keogh, and Lush (1937), in a recent monograph, which will be referred to later, have collected and analysed the available evidence on these questions, and have extended it with original observations. Recently Enders and Shaffer (1937) have observed a closely parallel series of phenomena in mixtures of Type III pneumococci and homologous antiserum. They observed a " dilution phenomenon ", *i. e.* neutral mixtures of pneumococci and antiserum became infective on dilution ; pneumococci which had remained in contact with an excess of serum for periods up to 65 hours were still infective after removal from the serum, and serum which had remained in contact with an excess of pneumococci for periods up to two weeks at 0° C. still contained protective antibody, though this was reduced after still longer periods of contact.

There is no reason to suppose, therefore, that the behaviour of viruses and their antisera is unique in these respects.

Until recently preparations of viruses used in work of this kind were necessarily impure, containing large and variable quantities of inactive material.

Craigie (1932) devised a method by which vaccinial elementary bodies could be obtained in very large quantities, practically free of tissue detritus and soluble impurities. He showed that these preparations were capable of absorbing the agglutinins and precipitins present in anti-vaccinial sera. The writer (Salaman, 1937), using Craigie's method of preparing elementary body suspensions with minor modifications, was able to remove all neutralizing antibody from anti-vaccinial sera by absorption with vaccinial elementary bodies. Very large doses were required to remove all neutralizing antibody, and it was suggested that previous failures to demonstrate absorption had been due to inadequate absorbing doses. It was pointed out that the dose of virus required to absorb all neutralizing antibody from a given quantity of serum was a high multiple of the dose which, when added to a similar quantity of serum, would give a neutral mixture.

It may be concluded from the evidence so far obtained that a mixture of vaccinia and antivaccinial serum which is just neutral when injected has the following characteristics :

1. A small part of the antibody is bound to the virus, and can be removed with it from the mixture.
2. The larger part of the antibody is free.
3. The virus when separated from this free antibody is still infective. (*Ad hoc* experiments convinced the writer that this infectivity does not depend on dilution of antibody. Agglutinated vaccinia from neutral serum-virus mixtures was deposited by centrifugation, and the supernatant fluid removed as cleanly as possible. The moist deposited virus was rubbed into scarified areas of rabbit's skin with a glass rod. Invariably it proved infective.)

Although it is clear that the condition of neutrality of a serum-virus mixture depends to a great extent on the presence of free antibody, the possibility that direct action on the virus may be a contributory factor has not been excluded. Indirect evidence of such action is provided by the progressive failure to recover infective virus by dilution or other methods as the period of incubation of

mixtures is lengthened. Of the abundant evidence of infectivity of virus recovered from serum-virus mixtures, none excludes the possibility that its infectivity may be considerably reduced. The present series of experiments was planned to test this possibility.

METHODS.

Preparation of Elementary Body Suspensions.

The strain of vaccinia used was the same as that described in a previous papers (Salaman, 1934, 1937). It was originally derived from sheep lymph, and had been passed serially 68 times on the shaved skin of rabbits. It will be called the B strain. In the latter paper the method of preparing elementary body suspensions by differential centrifugation was described, and this has been adhered to. The products showed a uniformly high degree of homogeneity and freedom from cellular detritus under the microscope. They were infective intracutaneously in the rabbit in dilutions of 1 in 10^6 to 10^8. They were stored for periods of two or three weeks at 0–4° C. without preservative. The suspending fluid was M/250 citric acid-disodium phosphate buffer solution pH 7·2. (For a more detailed description of these preparations see Macfarlane and Salaman, 1938.)

Preparation of Antisera.

Sera were obtained from rabbits following injections of infective vaccinia of the B strain. The special characters of each serum used will be described. Sera were filtered through Berkefeld V candles and stored at 0–4° C. without preservative.

Tests of Virulence.

Series of tenfold dilutions of the sample of virus to be tested were made in normal saline, using a clean pipette for each dilution. 0·2 c.c. of each dilution was injected intracutaneously.

Tests of Virus-neutralizing Power of Sera.

The " constant serum " method was used. A series of tenfold dilutions of an elementary body suspension was made in normal saline. Equal volumes of a constant dilution of the serum to be tested were added to each dilution of the virus. Control series of mixtures were made with normal serum and with an anti-vaccinial serum of known potency. The mixtures were allowed to stand for 1 to 2 hours at room temperature or at 37° C. (Variations of the temperature of incubation over this range do not, in the writer's experience, affect the result.) 0·2 c.c. volumes of each mixture were injected intracutaneously.

Titrations which were to be compared were always made on the same animal. Rabbits with firm, rather thick skins were chosen for all intracutaneous injections.

EXPERIMENTS.

Experiment I : Treatment of Elementary Bodies in a Wide Range of Dilutions with a Constant Dilution of Antiserum, followed by One Washing with Dilute Buffer Solution.

Virus : E.B. suspension B 68.

Sera : (i) " $PAVS_3$"—a pooled hyperimmune anti-vaccinial serum from a number of rabbits, each of which had received intracutaneous, followed by one or more intravenous injections of vaccinia, and were bled one week after the last injection.

(ii) " NRS "—Normal rabbit serum.

Eight mixtures were made :

a. Contained EB. suspension B 68 in original concentration	}	+ " $PAVS_3$" diluted 1 : 5.
b. ,, ,, ,, diluted 1 : 100		
c. ,, ,, ,, ,, 1 : 10,000		
d. ,, ,, ,, ,, 1 : 1,000,000		
a^1. ,, ,, ,, in original concentration	}	+ " NRS " diluted 1 : 5.
b^1. ,, ,, ,, diluted 1 : 100		
c^1. ,, ,, ,, 1 : 10,000		
d^1. ,, ,, ,, ,, 1 : 1,000,000		

The diluent was normal saline.

All the mixtures were incubated at 37° C. for 1½ hours. They were then centrifuged at 14,000 r.p.m. for ½ hour to deposit the E.Bs. The supernatant fluids were removed and kept for tests of virus-neutralizing power. The deposits were resuspended in the original volume of *M*/250 citric acid-disodium phosphate buffer pH 7·2, and centrifuged again at 14,000 r.p.m. for ½ hour. The supernatant fluids were removed and discarded, and the deposits resuspended, *a* and a^1 in the original volume, all the others in 1/10 of the original volume, of buffer solution.

Serial tenfold dilutions of each suspension were made in normal saline.

0·2 c.c. of each dilution was injected intracutaneously on one rabbit, and the lesions observed daily. The results are shown in Table I.

The first supernatant fluids from mixtures *a*, *b*, *c* and *d* were tested for virus-neutralizing power by the constant serum method, in comparison with a similar dilution of untreated antiserum. The results are shown in Table II.

Comparison of column *a* with column a^1, *b* with b^1, etc., in Table I shows that antiserum-treated E.Bs. gave a definitely lower end-titre and smaller individual lesions than normal serum-treated E.Bs. In the bottom row are given the sums of the cubes of the diameters of the lesions in each column (divided by 1000 for convenience). This cubic measure, which was used by Ledingham, Morgan, and Petrie (1931) for estimations of virus-neutralizing power of various fractions of anti-vaccinial serum, is roughly proportional to the volume of inflamed tissue. A comparison of these figures brings out strongly the difference between antiserum- and normal serum-treated elementary bodies.

TABLE I.—*Infectivity of E.Bs. Treated with Hyperimmune Serum PAVS$_3$.*

Diameters in millimetres of intracutaneous lesions on the 5th day after injection.

	Antiserum-treated.				Normal serum-treated.			
E.Bs. from mixture :	*a.*	*b.*	*c.*	*d.*	a^1.	b^1.	c^1.	d^1.
Undiluted	17				20			
$\times 10^{-1}$	13	10			19	17		
$\times 10^{-2}$	12	10	..	..	18	17	..	..
$\times 10^{-3}$	6	5	5	..	17	15	15	..
$\times 10^{-4}$	4	3	4	..	10	8	10	..
$\times 10^{-5}$	—	—	3	4	8	5	5	5
$\times 10^{-6}$	—	—	—	—	5	4	5	—
$\times 10^{-7}$	—	—	—	—	4	—	—	—
$\times 10^{-8}$	—	—	—	—	—	—	—	—
Sums of cubes of diameters $\times 10^{-3}$	9·1	2·2	0·2	0·06	27·3	13·9	4·6	0·12

TABLE II.—*Virus-neutralizing Power of Supernatant Fluids from Antiserum-Virus Mixtures.*

Diameters in millimetres of intracutaneous lesions on the 5th day after injection.

	+ Supernatant fluids from mixtures :				+ Unabsorbed serum $PAVS_3$ 1 : 5.	+ Normal rabbit serum, 1 : 5.
	a.	*b.*	*c.*	*d.*		
$\times 10^{-2}$	15	7	5	5	5	20
$\times 10^{-3}$	10	5	5	5	—	19
$\times 10^{-4}$	4	—	—	—	—	17
$\times 10^{-5}$	—	—	—	—	—	14
$\times 10^{-6}$	—	—	—	—	—	11
$\times 10^{-7}$	—	—	—	—	—	4

It will be seen that the quantity of virus inactivated by a given amount of serum depends on the quantity originally present. Although the figures do not warrant any exact formulation, they nevertheless fit, within the limits of experimental error, the hypothesis that a given amount of serum inactivates a constant percentage of any dose of virus with which it is in contact. Andrewes and Elford (1933) put forward a " percentage law " of this kind for the inactivation of bacteriophage, and Merrill (1936*a* and *b*) for that of the virus of equine encephalomyelitis, by antisera. Burnet, Keogh, and Lush (1937), to whose monograph reference should be made for further work on this point, consider that some such relation holds for a wide range of different virus-antiserum systems. In Experiment I it is at any rate evident that the serum in mixture *a* inactivated an amount of virus many times greater than that with which it was in contact in mixture *d*, yet the latter was not entirely inactivated.

Table II shows that the unabsorbed serum $PAVS_3$, in the same dilution as in the mixtures, is capable of neutralizing 100,000 M.I.D. in an ordinary virus-neutralization test. This means that mixture *a* contained an excess of virus, *b* was approximately neutral, *c* contained an excess and *d* a great excess of antiserum, as judged by this test. It shows, too, that there was a small but definite absorption of antibody in mixture *a*, but that it was inappreciable in the other mixtures. (Experience has shown that a much larger dose of virus than was present in mixture *a* is necessary to absorb all neutralizing antibody.)

"$PAVS_3$" had an agglutinin titre of 1 : 80 to 1 : 160. It is possible, therefore, to attribute the result of this experiment to agglutination of the elementary bodies by the antiserum. The smaller lesions as well as the lowered end-titre may be thought to have been due to clumping rather than to a true inactivation.

When the agglutinin, precipitin, and virus-neutralizing titres of the serum of rabbits are determined at various periods after vaccination, all three are found to rise together between the 5th and the 10th days ; after this there follows a slow decline of agglutinin and precipitin, but not of neutralizing titres, until, at a period varying between 10 and 15 weeks after vaccination, sera are obtained which have good virus-neutralizing power, but will not agglutinate elementary bodies or precipitate the specific soluble substance of the virus.

Table III is the record of agglutinin, precipitin, and virus-neutralizing titrations of the sera of two rabbits taken at various periods following intracutaneous vaccination.

TABLE III.

	Rabbit S50.			Rabbit S53.		
	A.	P.	N.	A.	P.	N.
Before vaccination .				$<2\cdot5$	$<2\cdot5$	<10
5 days after vaccination	$<2\cdot5$	$<2\cdot5$	<10 .	$<2\cdot5$	$<2\cdot5$	10
10 ,, ,, ,,	40	40	10^2 .	160	80	10^3
15 ,, ,, ,,	40	40	10^3 .	160	80	10^3
20 ,, ,, ,,	40	40	10^3 .	320	80	10^3
25 ,, ,, ,,	40	40	10^3 .	160	40	10^3
35 ,, ,, ,,	40	40	10^3 .	80	40	10^4
70 ,, ,, ,,	10	20	10^4 .	<10	<5	10^4
100 ,, ,, ,,	5	5	10^4 .	$<2\cdot5$	$<2\cdot5$	10^4

A. Reciprocals of agglutinin end-titres.

P. Reciprocals of precipitin end-titres. (For technique of agglutination and precipitation tests, see Salaman, 1937.)

N. Number of M.I.D. neutralized by 0·1 c.c. of 1 : 5 dilutions of the sera, as determined by the "constant serum" method.

It is not possible to do all the neutralization tests in such series on the same animal, and individual values must be regarded as approximate. But a number of other series of these tests have given similar results, and those recorded in Table III may be taken as representative of the general course of antibody titres following vaccination.

By the use of antisera having virus-neutralizing but negligible agglutinating power it should be possible to detect any direct inactivating effect on the virus not dependent on agglutination. Such sera were used in the following experiments:

Experiment II: Treatment of E.Bs. with a Virus-neutralizing but Non-agglutinating Serum, followed by One Washing with Dilute Buffer Solution.

Virus: E.B. suspension B 71.

Sera: (i) S26/90—an anti-vaccinial rabbit serum, collected 90 days after intracutaneous vaccination, which had virus-neutralizing but no agglutinating power.
(ii) N.R.S.—normal rabbit serum.

Four mixtures were made:

a. Contained E.B. suspension diluted	1 : 100	+ S26 diluted 1 : 5.
b. „ „ „ „	1 : 10,000	
a^1. „ „ „ „	1 : 100	+ N.R.S. diluted 1 : 5.
b^1. „ „ „ „	1 : 10,000	

The diluent was normal saline.

All the mixtures were incubated at 37° C. for 1½ hours, and then centrifuged at 14,000 r.p.m. for ½ hour. The supernatant fluids were removed and discarded. The deposits were resuspended in the original volume of the same buffer solution as was used in the first experiment, and again centrifuged at 14,000 r.p.m. for ½ hour. The supernatant fluids were removed and discarded, and the deposits finally suspended in 1/10 the original volume of buffer solution. Serial tenfold dilutions of each suspension were made in normal saline. 0·2 c.c. of each dilution was injected intracutaneously.

The virus-neutralizing power of S26/90 was tested on the same rabbit, by the " constant serum " method.

The results are shown in Tables IV and V:

TABLE IV.—*Virus-neutralizing Power of Antiserum S26/90.*

Diameters in millimetres of intracutaneous lesions on the 5th day after injection.

Dilutions of E.B. suspension	+ Serum 26 1 : 5.	+ Normal rabbit serum, 1 : 5.
$\times 10^{-1}$	17	21
$\times 10^{-2}$	16	18
$\times 10^{-3}$	16	18
$\times 10^{-4}$	7	17
$\times 10^{-5}$	—	15
$\times 10^{-6}$	—	13
$\times 10^{-7}$	—	6

TABLE V.—*Infectivity of E.Bs. Treated with Antiserum S26/90.*

Diameters in millimetres of intracutaneous lesions on the 5th day after injection.

		Antiserum-treated.		Normal serum-treated.	
	E.Bs. from mixture :	*a.*	*b.*	a^1.	b^1.
Dilutions of E.B. suspensions	$\times 10^{-1}$	13	..	22	..
	$\times 10^{-2}$	13	..	19	..
	$\times 10^{-3}$	10	7	17	15
	$\times 10^{-4}$	5	5	12	11
	$\times 10^{-5}$	..	3	7	6
	$\times 10^{-6}$	..	..	4	3
	$\times 10^{-7}$	..	..	..	..
Sums of cubes of diameters $\times 10^{-3}$		5·5	0·5	26·9	4·9

Comparison of column *a* with a^1 and *b* with b^1 in Table V shows that a definite reduction in infectivity was produced by treatment with this non-agglutinating serum.

A further possible criticism of these results concerns the washing of the elementary bodies after treatment with serum. Normal saline was not used as washing fluid because it agglutinates E.B. suspensions. The dilute buffer solution used does not have this defect, but it has the possible disadvantage that antibody may be insoluble in it. If this were so antibody might have been precipitated, thrown down in the centrifuge, and finally injected with the virus into the test animal, where it might have brought about the observed reduction in infectivity. In the following experiments normal rabbit serum diluted 1 : 10 with normal saline was used as washing fluid. The normal serum retards saline agglutination, so that it is inappreciable in the time taken for washing.

Experiment III : Treatment of Elementary Bodies in a Wide Range of Dilutions with a Constant Dilution of Virus-neutralizing but Non-agglutinating Serum, followed by One Washing with 10 per cent. Normal Serum in Saline.

Virus : E.B. suspension B 88.

Sera : (i) S53/100,—an antivaccinial serum collected 100 days after intracutaneous vaccination (see Table III).

(ii) N.R.S.—normal rabbit serum.

Eight mixtures were made in which the elementary body suspension and the sera were present in the same dilutions as in Experiment I. Incubation, centrifugation, and washing were carried out exactly as in that experiment, except that normal rabbit serum 1 : 10 in saline was used as the washing and final suspending fluid.

The results are shown in Table VI.

TABLE VI.—*Infectivity of E.Bs. Treated with Antiserum* S53/100.

Diameters in millimetres of intracutaneous lesions on the 5th day after injection.

E.Bs. from mixture:	Antiserum-treated.				Normal serum-treated.			
	a.	*b.*	*c.*	*d.*	a^1.	b^1.	c^1.	d^1.
Undiluted	19	..	..	..	20	..	..	..
$\times 10^{-1}$	18	16	..	..	18	18	..	..
$\times 10^{-2}$	17	14	..	..	18	17	..	..
$\times 10^{-3}$	17	14	12	..	18	17	17	..
$\times 10^{-4}$	16	13	11	..	18	17	17	..
$\times 10^{-5}$	13	7	7	7	16	16	13	11
$\times 10^{-6}$	9	6	—	7	14	12	11	8
$\times 10^{-7}$	4	—	—	—	9	7	6	5
$\times 10^{-8}$	—	—	—	—	5	—	—	—
Sums of cubes of diameters $\times 10^{-3}$	29·6	12·3	3·4	0·7	39·0	26·7	13·6	2·0

The results of the last two experiments (Tables V and VI) show that a definite reduction of infectivity may be effected by sera which have no appreciable agglutinating action, and that this reduction is not abolished by a single washing with a fluid in which antibody is certainly soluble. In Experiment III the reduction of infectivity, though less marked than in Experiment I, is quite definite and not inconsistent, within the limits of experimental error, with the "constant percentage" hypothesis. Incidentally the limitations of the method are clearly illustrated in this experiment, for the degree of inactivation shown (in the neighbourhood of 90 per cent.) is the least that can be detected with certainty by a method which depends on the intracutaneous injection of serial tenfold dilutions.

The washing of the serum-treated elementary bodies in the last three experiments was estimated to be sufficient to dilute any residual uncombined antibody well beyond its maximal effective dilution. It would be of theoretical interest to discover whether a return of infectivity could be brought about by further washing. The following experiment is an attempt to detect such a return:

Experiment IV: Treatment of Elementary Bodies with a Virus-Neutralizing but Non-agglutinating Serum, followed by Repeated Washing with 10 *per cent. Normal Rabbit Serum in Saline.*

Virus: E.B. suspension B 85.

Sera: (i) S 53/70—an anti-vaccinial serum collected 70 days after intracutaneous vaccination (see Table III).

(ii) N.R.S.—normal rabbit serum.

Two mixtures were made :

a. Contained E.B. suspension diluted 1 : 10 + S 53/70 diluted 1 : 2·5.
b. ,, ,, ,, + N.R.S. ,, ,,

The total volume of each mixture was 5 c.c. The diluent was normal saline.

Both mixtures were incubated at 37° C. for 1½ hours, and then centrifuged at 14,000 r.p.m. for ½ hour. The supernatant fluids were removed and the deposits resuspended in 5 c.c. of 10 per cent. N.R.S. in saline. 0·3 c.c. of each suspension was removed for titration of infectivity. The remainders were centrifuged at 14,000 r.p.m. for ½ hour. The supernatant fluids were removed and the deposits resuspended in 4·7 c.c. of 10 per cent. N.R.S. in saline. 0·3 c.c. of each suspension was removed for titration of infectivity. The remainders were centrifuged at 14,000 r.p.m. for ½ hour. The supernatant fluids were removed and the deposits resuspended in 4·4 c.c. of 10 per cent. N.R.S. in saline. 0·3 c.c. of each suspension was removed for titration of infectivity.

Serial tenfold dilutions of the six samples removed were made in saline. 0·2 c.c. of each dilution was injected intracutaneously. A control series of dilutions of untreated elementary bodies was also injected.

The results are shown in Table VII.

TABLE VII.—*Infectivity of E.Bs. Treated with Antiserum S53/70, after Successive Washings with 10 per cent. Normal Rabbit Serum in Saline.*

Diameters in millimetres of intracutaneous lesions on the 5th day after injection.

Dilutions of E.B. suspensions. After	Antiserum-treated. Washing. 1st.	2nd.	3rd.	Normal serum-treated. Washing. 1st.	2nd.	3rd.	Untreated E.Bs.
× 10^{-1}	15	15	16	21	21	19	..
× 10^{-2}	14	15	16	18	21	18	..
× 10^{-3}	14	14	15	17	18	18	17
× 10^{-4}	10	10	14	15	16	15	17
× 10^{-5}	4	6	8	11	15	13	14
× 10^{-6}	—	5	4	7	7	8	12
× 10^{-7}	—	—	—	4	5	5	4
× 10^{-8}	..	..	..	..	..	..	—
Sums of cubes of diameters × 10^{-3}	9·9	10·8	14·8	25·1	32·3	24·7	

After one washing the antiserum-treated E.Bs. show a reduction of infectivity of about 99 per cent. as compared with the normal serum-treated or the untreated E.Bs. Further washings appear to bring about some return of infectivity, but though the figures are suggestive they do not warrant a definite conclusion on this point.

DISCUSSION.

It has been shown that suspensions of vaccinial elementary bodies which have been treated with anti-vaccinial serum and then freed of uncombined serum are less infectious than control suspensions similarly treated with normal serum. This effect is not dependent on agglutination of the elementary bodies. The reduction of infectivity is always partial ; in no case has complete suppression of infectivity been observed, even when the original mixture contained a great excess of serum, as judged by the ordinary virus-neutralization test.

These results are consistent with the view that a given amount of antiserum inactivates a constant percentage of any dose of virus with which it is in contact, but their degree of accuracy is not high enough to warrant the formulation of an exact relation. Reference has been made to the work of Andrewes and Elford (1933) on various species of bacteriophage, and of Merrill (1936*a* and *b*) on the virus of equine encephalomyelitis. Both these workers record results which are closely analogous to those of the present investigation. No attempt was made by either to separate antigen and antiserum after interaction, but the technique of titration of the serum-treated phage and virus respectively involved serial tenfold dilution, so that the activity of any free serum must have been diluted out in almost all cases. Both workers found that a given amount of antiserum inactivates, under given conditions, an approximately constant percentage of any dose of the antigen upon which it acts. Andrewes and Elford further showed that " neutral " phage-antiphage mixtures contain a large excess of free antibody, and that a vast excess of phage, as compared with the "neutral" dose, is required to absorb this antibody (*cf.* p. 193). To explain the constant percentage inactivation they favoured the hypothesis that phage particles differ in their resistance to antiserum, but they were unable to isolate resistant strains. Merrill considers that aggregation of virus particles is the more likely explanation of the similar result which he obtained. Clifton, Mueller, and Rogers (1935), who confirmed the constant percentage inactivation of phage by antiserum, pointed out that the reaction has the typical characters of an adsorption. For an exhaustive account of these and other hypotheses the reader is referred to the monograph of Burnet, Keogh, and Lush (1937).

It is evident that neutralization of vaccinia in the presence of its antiserum cannot be wholly due to the direct effect on the virus, for when separated from the serum it is never completely non-infective. On the other hand, the direct effect must contribute to the total result—the non-infectivity of the mixture.

The free antibody may perhaps act by suppressing proliferation of the virus, or by rendering it more susceptible to phagocytosis ; further knowledge is needed to determine its rôle.

SUMMARY.

1. Suspensions of vaccinial elementary bodies suffer a reduction of infective titre when treated with anti-vaccinial serum. This reduction persists after all free serum has been removed.

2. The reduction of infectivity is not the result of agglutination of the elementary bodies.

3. The results recorded are consistent with the hypothesis, which has been advanced for other serum-virus systems, that under constant conditions a given quantity of antiserum inactivates a constant percentage of any dose of virus upon which it acts.

The writer wishes to express his thanks to Sir John Ledingham, F.R.S., for valuable advice and criticism.

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THE ARTIFICIAL OPSONIZATION OF BACTERIA. PART III.

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IN previous publications, Gordon and Thompson (1936, 1937) and Thompson and Gordon (1937), it has been demonstrated that tanning agents such as iron and chromium salts, vegetable tanning materials such as gallotannic acid and the commercial tannins, quebracho, mangrove, valonia, myrobalans, sumach, gambier, mimosa bark and chestnut wood, were capable of acting as artificial opsonins on *staphylococcus* and *B. typhosus*. It was further noted that the very basic protein, protamine, in the form of its sulphate, acted as an artificial opsonin on staphylococcus. In all cases there was a close correlation between agglutination of the organism by these substances and opsonization. Of all the materials examined the trivalent iron and chromium salts gave the most constant and easily reproducible results.

In a previous paper Gordon (1935) visualized the possibility that a protein might exhibit opsonic action. The opinion expressed in that paper was as follows : " Tannins of the ordinary type are not of course present in serum, but theoretically it is not impossible for one protein to function as a tanning agent on another protein, and this may possibly be an essential part of the mechanism of opsonization by serum either normal or immune."

The subsequent discovery that protamine sulphate will act as an opsonin justified the opinion then expressed. Protamine sulphate has not been employed as a tanning agent in the leather industry.

METHODS.

As is well known vegetable tannins can be largely removed from leather by prolonged washing with water, and the iron and chromium tanning agents from such leathers by treatment with solutions of salts of oxalic and tartaric acids or of other α-hydroxy acids. It was, therefore, deemed advisable to study the effects of washing on artificially opsonized organisms. For this purpose four opsonizing agents were used, namely :

0·5 per cent. iron ammonium alum.
0·5 per cent. chrome alum.
0·5 per cent. protamine sulphate (B.D.H. and Burroughs, Wellcome).
0·25 per cent. pure gallotannic acid.

These solutions were always used freshly dissolved in cold distilled water

and especially are these precautions important in the cases of chromium and iron salts. It was found that a 0·5 per cent. solution of gallotannic acid was unsuitable in use, as unless the organisms were washed very thoroughly after treatment there was always the danger of the leucocytes being damaged by the excess of tannin remaining.

Six different strains of *Staphylococcus aureus* were used in this series of experiments. A suspension of the organism in saline was prepared by washing the bacteria from agar slopes ; 1 c.c. quantities of this suspension were centrifuged and the deposited bacteria re-suspended in 1 c.c. quantities of the various solutions under test. The tubes were then allowed to stand for 3 hours on the bench. In all cases it was noticed that agglutination had occurred. The tubes were next centrifuged, the supernatant fluid pipetted off and the deposit of bacteria washed by varying methods of procedure, both in time of washing and the use of different solutions as will be detailed. After washing, the bacteria were finally re-suspended in normal saline. To a few drops of the bacterial suspensions was then added a drop of a washed leucocytic cream obtained by the method described in the previous papers (Gordon and Thompson, 1936). The tubes of leucocytes and bacteria were incubated for 1½ hours at 37° C., films were made and stained by Leishman's method. Control tubes of bacteria treated with saline alone were always set up.

With each opsonizing solution tested and for each strain of staphylococcus, ten tubes prepared as above were set up. Three different washing solutions were employed, 5 per cent. potassium oxalate, 5 per cent. potassium chloride and distilled water, and in addition two quick washings with normal saline were given in every case before the organisms were finally re-suspended in normal saline and the drop of leucocytic cream added.

Of the ten tubes used in each set of experiments the organisms were washed with 5 per cent. potassium oxalate solution in tubes numbered 1, 2 and 3 ; in tubes numbered 4, 5 and 6, the washings were carried out with 5 per cent. potassium chloride solution, and distilled water was employed for tubes numbered 7, 8 and 9. No. 10 tube served as a control where the organisms after opsonization only received the customary two final washings with normal saline to remove the possibility of damage to the leucocytes by either the opsonizing solution or the salt solutions employed for washing.

The organisms in tubes numbered 1 to 9 were all treated with 5 c.c. of their appropriate washing solution for 20 minutes, centrifuged and the supernatant fluid removed. Tubes numbered 2, 3, 5, 6, 8 and 9 were again treated with 5 c.c. of washing solution for 20 minutes, and during this time tubes numbered 1, 4 and 7 were given the two quick washings with saline described above. After this period of 20 minutes the six tubes enumerated above were centrifuged and the supernatant fluid removed. Tubes numbered 3, 6 and 9 again were treated with 5 c.c. of washing solution, whilst tubes numbered 2, 5 and 8 were being washed twice with saline. Finally tubes numbered 3, 6 and 9 were centrifuged, supernatant fluid removed, and given the two washings with saline. Thus in the procedure detailed tubes numbered 3, 6 and 9 had a time of contact with the washing solution of 60 minutes, tubes 2, 5 and 8 had 40 minutes, and tubes 1, 4 and 7 received 20 minutes' contact.

In all series an eleventh tube was set up, but this received no treatment

either with opsonizing agent or washing solution, and served as a control against spontaneous phagocytosis.

The different series of experiments were repeated many times, and although phagocytosis experiments do not lend themselves to definite quantitative measurements they nevertheless demonstrated clearly decisive trends.

RESULTS.

The artificial opsonization effect was removed with the 60 minutes period of washing in the cases of all four opsonizing agents, and with all the varying washing procedures, the control tube (No. 10) in which artificial opsonization had occurred and which received only the two rapid washings with saline always showing very marked phagocytosis. In all the experiments carried out the time periods of reversal vary somewhat, but 60 minutes of contact with the washing solutions (including distilled water) produced reversal in all cases.

With protamine sulphate and gallotannic acid, the opsonization was usually completely reversed by 40 minutes' washing with distilled water. Potassium oxalate and chloride both tended to hasten this reversal, the oxalate being rather more effective than the chloride. In fact distinct signs of reversal after 20 minutes' contact with solutions of these salts were observed.

With bacteria treated with iron and chromium salts, the reversal of opsonization by distilled water was always found to be more difficult than with protamine sulphate and gallotannic acid. Nevertheless reversal was complete after 60 minutes' contact with distilled water. Potassium oxalate was found to be the most effective washing agent for organisms opsonized by iron and chromium salts. In many cases reversal had occurred after 40 minutes' contact. The relative value as washing agents for iron and chrome treated bacteria arranged in order of their efficiency is potassium oxalate, potassium chloride and, lastly, distilled water.

Sodium carbonate is commonly employed in the leather industry for making chromium solutions basic, and so increasing their tanning effect. It was therefore added to the chrome alum solution used for opsonizing and it was noted that the agglutination of the organisms was intensified. In another set of experiments sodium bicarbonate was added after the chrome alum solution had been in contact with the bacteria for 3 hours, and in this manner the operation known as " neutralization " in the leather industry was simulated. It was found, however, that although agglutination was more marked, the degree of reversibility was substantially the same as before. When the artificial opsonizing agent was allowed to remain 24 hours (instead of the usual period of 3 hours) in contact with the bacteria, these were agglutinated in a more compact form, but again the reversibility was similar to that noted in the 3-hour period of contact between organisms and opsonizing agents. This last experiment is in harmony with the fact that tannin solutions in contact with gelatine jellies cause a rapid tanning of the surface, but the penetration is very slow. It follows from the above that artificial opsonization is mainly concerned with the actual surface of the organisms.

With a view to comparing the natural opsonic effect of serum with that of the artificial agents employed, similar experiments as regards washing to those already described were carried out, but 1 c.c. of serum was used instead of the artificial opsonins. It was found that reversibility did not occur to any appreciable extent with distilled water even after 60 minutes of contact. Whilst there was an appreciable diminution of phagocytosis after 60 minutes' treatment with solutions of potassium oxalate and chloride, it was evident that the effect of the serum had not been entirely removed. This would suggest that serum opsonization represents a firmer combination with the organism than is the case with the various artificial opsonins. With protamine sulphate the reversibility is paralleled by the action of protamine sulphate on insulin as it has been demonstrated that the insulin is slowly released from the insulin-protamine complex. Bischoff and Maxwell (1936) and Bischoff (1936) have recently shown a similar effect with tannic (gallotannic) acid and insulin. Here again we have noted a similar behaviour in the reversibility of opsonization.

DISCUSSION.

The relative ease of reversal of opsonization brought about by potassium oxalate on iron and chromium tanned organisms would appear to be due to the tendency of salts of these metals to form complexes with salts of oxalic, tartaric and other α-hydroxy acids. Such salts remove chromium from its combination with hide, and in fact such salts have been used commercially to regenerate hide from waste chrome leather so that it might be converted into glue and gelatine. In this connection it is interesting to recall that potassium chromioxalate, $K_3 [Cr (C_2O_4)_3]$ has no tanning effect upon skin and also will not bring about artificial opsonization, Gordon and Thompson (1936).

Another effect not yet considered is the lyotropic effect of salts in general upon proteins (Gordon and Thompson, 1933), where neutral salts produce dispersion of proteins without chemical decomposition such as accompanies the various methods of hydrolysis. The reversal effects of oxalate and chloride may therefore be due, to a large extent, to the dispersing power of the salts, but with oxalate there is in addition the complex-forming property which operates specially in the cases of organisms opsonized by iron and chromium salts.

SUMMARY.

A study has been made of the action of various degrees of washing upon staphylococci which have been opsonized by protamine sulphate, chrome alum, iron alum and gallotannic acid. The washing agents employed were 5 per cent. potassium oxalate, 5 per cent. potassium chloride, and distilled water, and these were applied for varying times.

Prolonged washing with distilled water sufficed to reverse the opsonization, but potassium oxalate was the most efficient agent in bringing about reversal, potassium chloride, however, being superior to distilled water. Serum opsonization was found to be not nearly so susceptible to washing as the various artificial opsonins.

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THE PRODUCTION OF STAPHYLOCOCCAL α-HÆMOLYSIN IN A CHEMICALLY-DEFINED MEDIUM.

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SINCE the well-known work of Walbum, Parker and Burnet on staphylococcal toxin, an extensive literature on the conditions necessary for its production has grown up. No standardized technique, however, has been used and the results since obtained have often been contradictory. In general they have added little to the work of the above authors. Apart from the necessity for free carbon dioxide and the beneficial effect of a semi-solid agar medium, definite correlation with the presence of a particular constituent of the medium used has rarely been satisfactorily established. In all cases complex media containing peptone or meat-extract have been employed, although attempts have been made to fractionate these substances chemically or by dialysis, adding them to media of known chemical composition. Walbum (1922) showed that hæmolysin for goat's cells was produced in Uschinsky's medium when augmented by the hexone base and other amino-acid fractions of Witte's peptone. Gengou (1935), using strains which produced little or no toxin on meat-extract alone, obtained good early production of toxin when 0·5 per cent. arginine was added. Proline, taurine, glycine and to a lesser extent ornithine, histidine, alanine, serine, oxyproline, glutamic acid, phenylalanine and tyrosine were also effective but much slower in their action. Aspartic acid, lysine, hydroxylysine, tryptophan, cysteine, methionine, valine, leucine and isoleucine

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were inactive. McLean (1937) obtained good yields of toxin in a cellophane-diffusate of Bigger's glycerol-veal-peptone medium without agar. Holt (1936) used a similar medium with added agar.

Glucose and other fermentable carbohydrates have been used with variable results. Bigger (1933) attributed the beneficial effect of glycerol in his medium to its effect on pH. Nélis (Nélis, 1933 ; Nélis, Bouckaert and Picard, 1934) found 0·1 per cent. glucose stimulated toxin production in a peptic-digest of veal medium. On the other hand, Parker, Hopkins and Gunther (1926) made every effort to exclude fermentable carbohydrates from the medium, and Forssman (1933) and Birch-Hirschfeld (1934) found that glucose was inhibitory. Schwartz (1935) stressed the importance of the hydrogen-ion concentration of the medium and showed that, when this was controlled, glucose had no effect on toxin production. He thus regarded the results obtained by other workers as entirely due to its effect on the pH of the medium.

With regard to the effect of inorganic salts on toxin production, the evidence is very conflicting. Walbum (1922) showed that no hæmolysin for goat's cells was produced in a simple peptone-broth medium when the magnesium was removed by precipitation. The addition of magnesium salts increased hæmolysin production. Calcium salts and high concentrations of sodium chloride and phosphate were inhibitory. Burnet (1930) added oxalate to his medium in order to remove calcium ions. On the other hand, Bigger (1933), using the hæmolysin for sheep's cells, was unable to obtain any effect with magnesium, calcium or sodium chloride when added to his glycerol-veal-peptone-agar medium, but stressed the need for a high phosphate-buffer concentration. Nélis, Bouckaert and Picard (1934) obtained no increase in toxin when magnesium or phosphate was added to their peptic-digest of veal medium. Gengou (1934) obtained a greater yield of toxin in the presence of 0·5 per cent. sodium chloride. Forssman (1934) found that the inorganic requirements for hæmolysin production depended on the strain of staphylococcus used. One strain produced more hæmolysin when sodium chloride was added and less in the presence of magnesium. With other strains the reverse was the case. Calcium salts were not invariably inhibitory (Forssman, 1933).

The observation of Parker *et al.* (1926) that growth in the presence of 10 per cent. carbon dioxide increased toxin production has been confirmed by most workers. Burnet (1930) found a difference between *Staphylococcus aureus* and *Staphylococcus albus* strains in this respect. The former required carbon dioxide for toxin production, the latter did not. Bigger (1933), however, using Burnet's strains found no difference between them. By using a well-buffered medium containing a fermentable substance (glycerol) he was able to dispense with the need for carbon dioxide and regarded its effect as being explained solely by its action on the hydrogen-ion concentration of the medium. With a few strains, carbon dioxide was definitely prejudicial to hæmolysin production. Since he used sheep red blood-corpuscles throughout, his results were concerned mainly with β-hæmolysin.

Less consideration has been given in the literature to the question of oxygen tension. Burky (1933) found that no hæmolysin was produced in broth cultures under a vaseline seal, but stated that necrotoxin was unimpaired. Casman 1 938) obtained good yields of toxin in a medium subjected to constant aëration.

He (private communication) used a proteose-peptone-veal infusion and aërated with 80 per cent. oxygen and 20 per cent. carbon dioxide. Similar results were obtained with a yeast-extract medium. Holt (unpublished) found that oxygen was an important factor in toxin production, but he attributed it mainly to an effect on growth.

The diversity of the results collected above may be due largely to technical differences. When crude substances from natural sources such as peptone or meat-extract have to be included in the basal medium, such differences are to be expected. Recently a medium has been obtained composed entirely of chemically-known substances on which *Staphylococcus aureus* grows well (Fildes, Richardson, Knight and Gladstone, 1936; Knight, 1937). A possibility of investigating the requirements for toxin production under rigidly controlled conditions was thus presented. Further, if the organism produced toxin in a synthetic medium, the isolation and purification of toxin would be simplified. A clinical application might also be found in the production of toxoid free from substances giving secondary toxic symptoms.

Preliminary work showed that little *a*-hæmolysin was produced in the complete amino-acid medium of Fildes *et al.* (Gladstone, 1937). In the present paper an account is given of modification in the medium whereby yields of hæmolysin* comparable with those obtained from standard media used in toxin production can be obtained.

TECHNIQUE.

Medium.

As a basis the medium of Fildes *et al.* (1936) as modified by Gladstone (1937) was used. It contained, besides inorganic salts, 16 amino-acids, glucose aneurin and nicotinamide. Further modifications are given subsequently.

pH.—The medium contained *M*/30 phosphate buffer at pH 7·6. Under the conditions of incubation (see below) the pH fell to 7·0.

Strain and inoculum.

In order to test the effect of the constituents of the medium on hæmolysin production, it was necessary to exclude as far as possible their effect on growth. For this purpose it was desirable to use a strain which had the least exacting growth requirements, but which under simple conditions of growth kept its toxin-producing abilities unimpaired. Recently it was possible to obtain such a strain. A very stable toxin-producing strain of *Staphylococcus aureus*, A_γ (Bigger, 1933), was kindly supplied by Dr. J. W. Bigger. By a process of "training" it has been possible to grow this strain on a simple chemically-defined medium whose main source of nitrogen was ammonia (Gladstone, 1937). On this medium no hæmolysin was produced, but it maintained its ability to produce hæmolysin when transferred to an adequate medium.†

* In this paper where the term "hæmolysin" appears unqualified by prefix, "*a*-hæmolysin" is to be understood.

† A slight impairment in toxigenicity was observed after two years. By plating and selecting colonies, the toxigenicity of the strain was maintained.

The inoculum used throughout was taken from this "adapted" strain growing in the simplified medium. It was subcultured every 14 days and kept at room temperature after 3 days' growth at 37° C. The inoculum was 0·06 c.c. of culture per 20 c.c. of medium.

Preparation of a-hæmolysin.

Cultures for hæmolysin production were grown in 20 c.c. of medium in 100 c.c. Erlenmeyer flasks in an atmosphere of 20 per cent. CO_2 in air. In later experiments air was replaced by O_2. Unless otherwise stated, growth was allowed to continue for 6 days. The organisms were then centrifuged and the supernatant removed and tested for hæmolysin.

a-hæmolysin tests.

In the majority of cases estimations of the *Lh* dose were made to the nearest 0·05 c.c. Small quantities of hæmolysin were tested for their M.H.D.

Red blood-corpuscles were taken from a single rabbit. Recent work confirmed the justification for this precaution (Timmerman, 1937). Bleedings were made once per week. The cells were washed three times with 0·9 per cent. saline and stored in a 50 per cent. dilution in the ice-chest. A 2 per cent. suspension was used for testing.

Antitoxin.—A sample of antitoxin containing 909 International Standard Units per 1 c.c., was kindly supplied by Miss Llewellyn Smith. It was tested periodically against a standard preparation. No loss of potency was detected after a year.

EXPERIMENTAL.

Hydrolysed Proteins and Amino-acids.

From the work of Gengou (1935) it appeared that amino-acids played an important part in hæmolysin production. Most of the amino-acids used by him, however, were present in Fildes' basal medium in which little hæmolysin was produced (*Lh* dose $>$ 1·0 c.c., M.H.D. between 0·06 and 0·03 c.c.). It was thought possible that others might be required or that the quantity of each was important. As a preliminary, a comparison was made of the effects of acid-hydrolysates of different proteins on hæmolysin production. The following proteins were used: gelatin, egg-albumen, edestin, casein, fibrin, peanut-protein, wool-protein, zein, gliadin, cocksfoot-grass-protein, whole serum and Difco bacto-peptone. All except peptone were hydrolysed in a concentration of 5 per cent. with 2·5 *N* H_2SO_4 for 40 hours. Peptone was hydrolysed with 2 *N* H_2SO_4 for 12 hours. The sulphuric acid was removed with barium hydroxide. The hydrolysed proteins were added to the amino-acid basal medium in 1 per cent. and lower concentrations. Of these edestin was the most effective, giving in a 1 per cent. dilution an *Lh* dose between 0·15 and 0·1 c.c. and a M.H.D. of less than 0·002 c.c. Cocksfoot-protein, fibrin and peptone were also effective. Through the kindness of Prof. A. C. Chibnall 500 g. of highly purified crystalline edestin was obtained. When hydrolysed this gave materially the same results as

the less pure sample. The indication was, therefore, that an amino-acid mixture corresponding to that of hydrolysed edestin would be most suitable for hæmolysin production. Table I gives a summary of the amino-acid content of edestin as far as it is known.

TABLE I.—*The Amino-acids of Edestin.*

Amino-acid.	Quantity present.		Authority.*
	Per cent.	Molarity.	
Glycine	3·8	0·505	Abderhalden (1903).
Alanine	3·6	0·41	,,
Serine	0·33	0·031	,,
Oxyproline	2·0	0·153	,,
Valine	5·6	0·478	Levene and Van Slyke (1909).
Leucine, *iso*leucine, etc.	8·1	0·618	,,
Phenylalanine	3·09	0·188	Osborne and Liddle (1910).
Proline	4·1	0·356	,,
Tyrosine	4·5	0·247	Folin and Ciocalteu (1927).
Tryptophan	1·5	0·073	,,
Cystine	1·25	0·052	Vickery and White (1933).
Methionine	2·07	0·139	Baernstein (1932).
Aspartic acid	10·2	0·766	Jones and Moeller (1928).
Glutamic acid	19·2	1·30	,,
Lysine	2·19	0·15	Vickery and Leavenworth (1928).
Arginine	15·8	0·908	,, ,,
Histidine	2·08	0·134	,, ,,
Ammonia	2·28	1·34	Osborne and Harris (1903).
	91·69		

* I am indebted to Dr. G. M. Richardson for these references.

A mixture of amino-acids was made up as in Table I. In order to obtain equivalent concentrations of optically active amino-acids, where these acids were synthetic double the concentration was used. Such amino-acids were alanine, serine, valine, leucine, phenylalanine, methionine, aspartic acid and lysine. This mixture was "acid hydrolysed" in the same way as whole edestin, to make the conditions comparable, and added to the basal amino-acid medium. Table II gives the results of the hæmolysis test when the organism was grown on this medium.

From Table II it is seen that a synthetic mixture of amino-acids was much inferior to edestin for producing hæmolysin. The significance of this result is considered later. It was, however, considerably better than the basal amino-acid medium alone. With the exception of serine, which was found to have no influence on hæmolysin production in the quantity present in edestin, all the known amino-acids of edestin were present in the basal medium. It was,

therefore, a question of quantity. Numerous experiments were made with a view to determining which amino-acids were necessary and what quantity of each was required. The work was complicated by the fact that many appeared to be interdependent. Moreover, those apparently stimulating toxin production in one concentration became inhibitory if the concentration was increased. Using as a basis the quantities present in 1 per cent. edestin, the most important amino-acid was soon found to be arginine. This was in accordance with the results obtained by Gengou (1935). Glycine and to a lesser extent proline also affected hæmolysin production. In the complete absence of all three of these acids, the *Lh* dose increased from 0·4 to > 1·0 c.c. when grown in the " synthetic edestin " medium. From Table I it can be seen that 1 per cent. edestin contained 0·158 per cent. arginine (*M*/110), 0·041 per cent. proline (*M*/280), and 0·038 per cent. glycine (*M*/198). The basal amino-acid medium contained only 0·004 per cent. arginine (*M*/4000), 0·008 per cent. proline, (*M*/1500) and 0·005 per cent. glycine (*M*/1500). Table III shows the effect of adding these three amino-acids in the quantities contained in 1 per cent. edestin to the amino-acid basal medium from which they had been excluded.

TABLE II.—*The Effect of Growth in a Mixture of Amino-acids, Equivalent to the Amino-acid Content of Edestin, on the Production of a-hæmolysin.*

		Per cent.	*a*-hæmolysin (*Lh* dose in c.c.).
Basal medium (Fildes *et al.*, 1936; modified by Gladstone, 1937)	Alone	..	>1·0
	+ hydrolysed edestin	1·0	0·15
	,, ,,	0·2	0·2
	,, ,,	0·04	0·9
	+ amino-acid mixture equivalent to hydrolysed edestin	1·0	0·4
	Ditto	0·2	0·4
	,,	0·04	>1·0

TABLE III.—*The Effect of Arginine, Proline and Glycine on a-hæmolysin Production.*

Quantities equivalent to 1 per cent. edestin.

Amino-acid basal medium lacking arginine, proline and glycine				*a*-hæmolysin (*Lh* dose in c.c.). Time when harvested (in days): 4	6
Ditto	+ proline (*M*/280)			>1·0	>1·0
,,	—	+ glycine (*M*/198)		>1·0	0·9
,,	—	—	+ arginine (*M*/110)	0·7	0·4
,,	+	+	—	>1·0	0·8
,,	+	—	+	0·5	0·35
,,	—	+	+	0·4	0·3
,,	+	+	+	0·25	0·2

In Table III the importance of arginine and glycine is clearly shown. Proline was less important. It can be seen that the quantity of hæmolysin produced in the amino-acid basal medium when augmented with arginine, glycine and proline in the concentrations present in 1 per cent. edestin actually exceeded that produced in the " synthetic edestin " mixture. This was found to be due to the inhibitory effect of the concentration of leucine ($M/162$)* present in the latter. When this was removed the amount of hæmolysin produced in the " synthetic edestin " mixture was comparable to, but did not exceed, that obtained from the basal medium with the necessary increased quantities of arginine, proline and glycine. The other amino-acids, therefore, appeared to be present in the basal medium in adequate concentrations, if, indeed, they were required at all.

Concentration of arginine, proline and glycine.

To test whether the concentration of arginine, proline and glycine present in 1 per cent. edestin was the optimum for hæmolysin production, each of these amino-acids was titrated in the basal amino-acid medium in the presence of this concentration of the other two. The optimum concentration for arginine was found to be between $M/50$ and $M/150$ (equivalent to 2·2 to 0·7 per cent. edestin). Quantities less than $M/300$ and greater than $M/25$ inhibited toxin production. Gengou (1935) used 0·5 per cent. arginine ($M/35$) but found quantities greater than this inhibitory. He attributed inhibition to a decrease in H-ion concentration. The pH of the culture after growth in the amino-acid medium with quantities of arginine, proline and glycine equivalent to 1 per cent. edestin was about 6·6. When the arginine was increased to $M/25$ this rose to about 7·4. Some inhibition was still apparent, however, when this rise in pH was controlled by the periodic addition of HCl during growth. Proline also showed an optimum concentration between $M/140$ and $M/700$ (equivalent to 2·0 to 0·4 per cent. edestin). With glycine no upper limit was obtained, but hæmolysin production diminished below a concentration of $M/500$ (equivalent to 0·4 per cent. edestin). It would seem, therefore, that the concentrations of these amino-acids equivalent to 1 per cent. edestin are within the optimum range for hæmolysin production and these quantities ($M/110$ arginine, $M/280$ proline and $M/198$ glycine) were subsequently used throughout.

The necessity for other amino-acids.

Arginine, proline and glycine in their optimal concentrations were added to a basal medium from which all other amino-acids except cystine were excluded. An ammonium salt was added to keep the nitrogen content constant. The organism growing in such a medium produced no hæmolysin (*Lh* dose > 1·0 c.c.); other amino-acids were therefore required. After preliminary experiments in which amino-acids were added singly and in pairs without result, each amino-acid was omitted in turn from the complete amino-acid basal medium augmented with the requisite quantities of arginine, proline and

* This concentration of leucine was apparently without effect in the hydrolysed edestin medium.

glycine. Owing to slight variations in the quantities of hæmolysin produced, the experiments were performed in duplicate.

In the complete medium the only amino-acid materially affecting hæmolysin production was phenylalanine. Oxyproline was not required, hæmolysin being if anything increased in its absence. When leucine was omitted, valine and possibly glutamic acid showed some effect and with the removal of aspartic acid, alanine appeared effective. Tyrosine, histidine, glutamic acid and lysine, however, as well as oxyproline, leucine and aspartic acid could be omitted without affecting hæmolysin production very greatly. The organism was then growing on a mixture containing arginine, glycine, alanine, proline, valine, phenylalanine besides cystine, tryptophan and methionine. On this medium the removal of any one of the first six amino-acids had greater effect on hæmolysin production than when growing on the complete medium. In the absence of arginine or glycine, no hæmolysin (*Lh* dose $> 1{\cdot}0$) was produced; without phenylalanine, the *Lh* dose was 0·95 c.c.; without valine, 0·7 c.c.; without proline 0·5 c.c.; without alanine, 0·4 c.c. With all present, it was 0·3 c.c., compared with 0·2 c.c. in the complete medium. It would seem that, with the exception of arginine and glycine, these amino-acids could be to some extent replaced by others of the complete medium. Attempts to increase the concentration of these amino-acids did not increase toxin production, but often lead to inhibition. In general these results are in accordance with those obtained by Gengou (1935); oxyproline and serine, however, were found by him to stimulate toxin production and valine appeared to be inactive possibly because it was present in his basal medium (meat-extract).

Tryptophan and methionine.—Although generally included in the medium, neither of these acids had any effect on hæmolysin production.

Cystine.—To test whether hæmolysin production is affected by omitting cystine from the medium, another organic source of sulphur, *e. g.* mercaptoacetate, must be included for growth to occur (Fildes and Richardson, 1937). As this is less readily used by the organism, its initial concentration was made equal to ten times that of the cystine. The results obtained are given in Table IV.

TABLE IV.—*The Effect of Cystine and Sodium Mercaptoacetate on a-hæmolysin Production.*

	Concentration.	Growth. Days: 1	3	6	*a*-hæmolysin (*Lh* dose in c.c.).
Cystine	*M*/1000	++	+++	++	0·4
,,	*M*/5000	++	+++	++	0·2
,,	*M*/25,000	+	+++	++	0·25
,,	*M*/125,000	+	+++	++	0·4
Sodium mercaptoacetate	*M*/100	+	+++	++	0·35
,, ,,	*M*/500	tr	+++	++	0·55
,, ,,	*M*/2500	0	++	++	>1·0
,, ,,	*M*/12,500	0	+	+	>1·0

Note.—The *plus* signs refer to amount of growth as judged by opacity measurements.

In spite of the slight differences in growth, there is little doubt that cystine was considerably more effective than mercaptoacetate for the production of α-hæmolysin. The optimum concentration of cystine was about $M/5000$, which is the quantity normally present in the amino-acid medium. With quantities greater than this some inhibitory effect was apparent.

Ornithine.—When arginine was replaced by an equimolecular ($M/110$) concentration of ornithine, the *Lh* dose of the α-hæmolysin produced in the medium was increased from 0·2 c.c. to 0·4 c.c. Ornithine will thus replace arginine to some extent, but is less effective. This is in accordance with the results obtained by Gengou (1935). Greater concentrations of ornithine were inhibitory probably due to the consequent increase in H-ion concentration; with a concentration of $M/110$ the pH of the hæmolysin was about 5·5 (just red to methyl-red). A mixture of guanidine and ornithine in the same molecular concentration as arginine gave the same result as ornithine by itself. Such a mixture will, therefore, not wholly replace arginine.

Hydroxyglutamic acid, dl-trans-β-*hydroxy-amino-butyric acid* (*threonine*), iso*leucine*, nor*leucine*, β-*alanine*.—None of these amino-acids had any effect on hæmolysin production when tested in concentrations of $M/500$.

The effect on growth of omitting amino-acids apparently necessary for hæmolysin production.

Reference to earlier work (Gladstone, 1937) will show that most of the amino-acids necessary for early growth of strain Aγ (strain 22) were those amino-acids which, in the present work, were found to have an influence on hæmolysin production. As previously stated, attempts were made to obviate this difficulty by using a strain adapted to grow in the absence of all amino-acids. In spite of this precaution, however, when arginine or glycine was absent there was an obvious difference not only in the rate, but in the amount of growth. In the absence of other amino-acids little difference was apparent. A much greater concentration of arginine and glycine was required for hæmolysin production than for maximum growth. Their effect on hæmolysin production cannot, therefore, be explained by their effect on growth.

Nitrogen compounds other than amino-acids.

Creatine, guanine and guanidine had no effect on hæmolysin production.

A constituent of hydrolysed edestin stimulating α-hæmolysin production.

It has been stated that a mixture of amino-acids in the proportions in which they occur in edestin was less effective than hydrolysed edestin itself in producing hæmolysin (Table II). It was pointed out that the concentration of leucine in such a mixture was somewhat inhibitory. That it did not seem to be so in the hydrolysed edestin medium suggested that some protective substance was also present. Even when leucine was omitted, however, the synthetic mixture was still inferior to the hydrolysed edestin medium.

To find out whether hydrolysed edestin had a stimulating effect on hæmolysin production not due to the known amino-acids that it contained, concentrations of 0·004 to 0·5 per cent. were added to a chemically-controlled medium

optimum for the production of hæmolysin (*i. e.* the amino-acid medium of Fildes modified by the addition of arginine *M*/110, glycine *M*/198 and proline *M*/280). Concentrations of 0·02 and 0·5 per cent. increased hæmolysin from *Lh* 0·25 c.c. to *Lh* 0·15 c.c. and 0·1 c.c. respectively.

With the object of ascertaining whether this constituent was an amino-acid, acid-hydrolysed edestin was precipitated with mercuric acetate-sodium carbonate (Neuberg and Kerb, 1912). The filtrate, freed from mercury and as much of the acetic acid as possible, contained 1·92 mg. of amino-nitrogen per 1 c.c. and still had a slight stimulating action on hæmolysin production. When the amino-nitrogen was removed by treatment with nitrous acid, this effect was not lost, but an inhibitory substance was also present which became evident in concentrations greater than 0·1 per cent. This was probably due to acetate (from the Neuberg-Kerb precipitation), all of which had not been removed.

These results suggest that the constituent of edestin stimulating hæmolysin production in a medium which contained the optimum concentrations of amino-acids did not contain a primary amino-group. The effect was shown not to be due to inorganic ions.

The Effect of Inorganic Salts.

Magnesium, iron and phosphate ions.

Alteration in the concentration of the inorganic salts present in the medium (magnesium sulphate *M*/6000, ferrous ammonium sulphate *M*/20,000 and phosphate buffer *M*/30) had no enhancing effect on hæmolysin production. In the absence of both magnesium and iron, no hæmolysin (*Lh* $>$ 1·0 c.c.) was produced ; growth was slightly impaired, but not enough to account for the loss of hæmolysin. Magnesium and iron were to some extent interchangeable, but the best yields were obtained when both were present in the above concentrations.

Calcium ions.

Calcium ions were found to have an appreciable inhibitory effect on hæmolysin production, confirming Walbum's (1922) observation. Owing, however, to the presence of phosphate, when soluble calcium salts were added to the medium calcium phosphate was precipitated. Only very small concentrations of calcium ions could, therefore, be present.

Other inorganic salts, such as sodium chloride, potassium nitrate, hay ash, etc., were also tested. No stimulating effect on hæmolysin production was found. In certain concentrations most were inhibitory.

The Effect of Carbon and Energy Sources.

Glucose.

The main source of energy in the amino-acid medium was glucose (*M*/80). When this was omitted, growth was impaired and the quantity of hæmolysin produced fell from *Lh* 0·2 to *Lh* 0·8 c.c. The optimum concentration of

glucose was found to be about $M/80$. In concentrations greater than $M/30$, no hæmolysin (*Lh* > 1·0 c.c.) was found. This was at first attributed to the fall in pH. When this was controlled during growth, hæmolysin production was still inhibited. It would seem, therefore, that the inhibitory effect of these concentrations of glucose was not entirely due to the increase in the hydrogen-ion (*cf.* Schwartz, 1935). It was probably caused by the accumulation of products of glycolysis.

Other sugars and polyhydric alcohols.

When glucose was replaced by equimolecular concentrations of other sugars and polyhydric alcohols, the following results were obtained: with mannose, galactose or lactose the *Lh* dose of hæmolysin was 0·2–0·25 c.c.; with maltose and inositol, it was 0·3 c.c.; with glucosamine, 0·45 c.c.; with xylose, arabinose and erythritol, 0·5 c.c.; with glycerol, 0·6 c.c.; with rhamnose, 0·85 c.c. and with mannitol 0·9 c.c. In the presence of glucose, the *Lh* dose was 0·2 c.c. and without added carbon or energy source, it was 0·8 c.c. None of these substances therefore was better than glucose. The inhibition in the presence of mannitol was probably due to the low pH (6·0). Contrary to the results of Bigger (1933), glycerol was less effective than glucose. Hæmolysin production was not increased when combinations of these substances were used.

Organic acids.

Glucose was replaced by sodium salts of each of the following organic acids in an equimolecular concentration: lactic, pyruvic, oxalacetic, malic, succinic, fumaric, malonic and maleic acids. The experiment was performed in two series: in the first the pH was made equal throughout growth to that of a culture containing glucose, by the periodic addition of HCl, brom-thymol-blue being present in the medium as an indicator of the H-ion concentration. In the second series the pH was not controlled. In both series the replacement of glucose by salts of organic acids lead to impairment of growth and poor production of hæmolysin. Only sodium succinate and sodium malate appeared to have any beneficial effect and both were inferior to glucose. In general, better yields were obtained in the series in which the pH was controlled.

The Effect of Carbon Dioxide.

A well-marked "CO_2-effect" was obtained when the organism was grown in the chemically-defined amino-acid medium. The carbon dioxide tension used by Burnet (20 per cent.) was found to be most effective. In air the *Lh* dose was > 1·0 c.c.; in 20 per cent. CO_2 + 80 per cent. air or O_2 it was 0·2 c.c. The effect was shown not to be due to its effect on the H-ion concentration of the medium. This is in accordance with Burnet's findings with *Staphylococcus aureus* strains, but is contrary to those of Bigger (1933) who used the same strain (Aγ). However, as previously mentioned, his findings apply more to β-hæmolysin.

The Effect of Oxygen.

In the chemically-defined amino-acid medium, oxygen was an important factor, as shown by the following results:

Relation between volume of medium and exposed surface area.

Table V shows the necessity for an adequate exposed surface area to produce optimal amounts of hæmolysin in a given quantity of medium. When the volume of medium in c.c. was greater than the surface area in sq. cm., hæmolysin production was impaired and seemed to be approximately proportional to the ratio between the exposed surface area and volume of the medium. On the other hand there was an indication that the surface area could be too great, as shown by the results obtained in a Petri dish (Table V).

TABLE V.—*The Relation Between the Exposed Surface Area and the Volume of the Medium in the Production of a-hæmolysin (grown in 20 per cent. CO_2 + 80 per cent. O_2).*

Culture vessel.	Exposed surface area of medium (sq. cm.).	Volume of medium (c.c.).	Time when harvested in (days):	*a*-hæmolysin (*Lh* dose in c.c.). 3	6
250 c.c. bottle	28·2	10		0·3	0·2
,,	28·2	20		0·3	0·2
,,	28·2	40		0·4	0·3
,,	28·2	80		0·7	0·6
,,	28·2	120		1·0	0·8
Test-tube (8 × 1¼ in.)	8·0	10		0·3	0·2
,, ,,	8·0	20		0·4	0·4
,, ,,	8·0	40		1·0	0·9
,, ,,	8·0	60		>1·0	1·0
Test-tube (6 × ¾ in.)	2·8	10		0·8	0·7
,, ,,	2·8	20		>1·0	>1·0
100 c.c. flask	24·0	20		0·3	0·2
Petri-dish (4½ in. diam.)	99·0	20		0·3	0·4

Owing to changes in oxygen supply, slight differences in growth were observed, but not sufficient to account entirely for differences in hæmolysin production. The amount of hæmolysin produced in cultures in which the ratio of surface area to volume of the medium was low, was less constant than that in cultures grown under optimum conditions, and this was also found when 80 per cent. air was used in place of oxygen and when the culture flasks were packed into gas-jars without precautions to keep them upright. The best yields of hæmolysin were obtained when the gas in the culture vessels was renewed daily and the growth kept in suspension by gently shaking the cultures periodically.

Selective production of hæmolysin in the part of the medium in contact with oxygen.

1 per cent. ash-free agar was added to 80 c.c. of amino-acid medium contained in a large test-tube. The medium was inoculated before setting and a shake-culture made. A similar culture was put up containing $M/20{,}000$ methylene-blue. Growth occurred uniformly throughout the cultures. After 6 days the depth of medium in the methylene-blue culture still remaining blue was measured (about the upper 5 cm.). At a point 5 cm. from the surface of the agar in the other culture, the tube and the contained medium were cut through and a further division was made half-way down the tube from this point. The agar was solid enough to allow a neat division to be made. The three sections of medium, each containing about 25 c.c., were chopped up and centrifuged rapidly. About 3 c.c. of clear supernatant fluid were obtained from each. When tested for hæmolysin, the upper section contained *Lh* dose of 0·45 c.c.; in the other two sections the *Lh* dose was $>$ 1·0 c.c. Thus hæmolysin production was confined to that part of the medium in which oxygen kept the redox potential more positive than about *Eh* + 0·01 volts.

The effect of redox indicators as possible oxygen carriers.

Holt (private communication) was unable to obtain augmented toxin production when redox indicators covering a wide range of potential were added to broth cultures. The tests reported in this paper were carried out in test-tubes containing 20 c.c. of medium; the oxygen supply was thus sub-optimal and toxin production was impaired (*Lh* $>$ 1·0 c.c., M.H.D. 0·03 c.c.). Using pyocyanine, phenazine methosulphate, thiochrome and phenol-indo-2 : 6-dibrom-indophenol in a concentration of $M/1 \times 10^{-5}$ and methylene-blue in a concentration of $M/1 \times 10^{-6}$, a slight indication of an increase in hæmolysin production was obtained (*Lh* 1·0 c.c., M.H.D. 0·015 c.c.). Lacto-flavine, phenosafranine and brilliant cresyl-blue were inactive or inhibitory in similar concentrations. That the beneficial effect of these substances was due to their acting as oxygen carriers could not be ascertained with certainty, but was suggested by their having less effect when the cultures were grown in conical flasks in which oxygenation was more effective.

The effect of anaërobic conditions of growth on hæmolysin production.

When grown under anaërobic conditions in the amino-acid medium with the necessary quantities of uracil and pyruvic acid (Richardson, 1936) in an atmosphere of 20 per cent. CO_2 in hydrogen with rigid precautions to exclude oxygen, very small quantities of hæmolysin (*Lh* dose $>$ 1·0 c.c., M.H.D. 0·06 c.c.) were produced. The effect was not due to impairment of growth as this was little different from an aerobic culture. Increasing the concentration of pyruvate in the medium (from $M/3000$ to $M/130$ and $M/26$) or the addition of potassium nitrate ($M/400$) had no effect on hæmolysin production.

Continuous aëration with 20 per cent. CO_2 + 80 per cent. oxygen.

Cultures grown under these conditions, in spite of the increased growth, were completely devoid of hæmolysin (contrast Casman, 1938). After 6 days the *Lh* dose was > 1·0 c.c. and the M.H.D. was > 0·5 c.c. When tested after 24 hours, a M.H.D. of 0·25 c.c. was obtained. The hæmolysin was apparently destroyed soon after it was formed. With a view to explaining this result, the effect of gases on preformed hæmolysin was investigated.

The Effect of Gases on Preformed Hæmolysin.

Oxygen (80 per cent.) + carbon dioxide (20 per cent.).

When sterile (Seitz-filtered) hæmolysin was gassed with 20 per cent. CO_2 in oxygen for 2 hours, complete destruction of hæmolysin occurred. It had not only lost its hæmolytic power, but also its combining power with antitoxin, *i. e.* it had not been converted to toxoid. This destruction was at first attributed to excessive oxygenation. Todd (1934) found no evidence of destruction when toxin was incubated in air for 5 days at 37° C., but he did not record any aëration experiments.

To test whether oxygen was responsible for the destruction of hæmolysin described above, a Seitz-filtrate of a culture containing hæmolysin was shaken in a Warburg vessel for 2 hours at 37° C. in oxygen containing 5 per cent. carbon dioxide. No loss of hæmolysin was observed. It would seem that oxygen was not responsible for the destructive effect.

Nitrogen (free from oxygen).

Nitrogen from a cylinder was freed from oxygen by passing it over red-hot copper gauze. The gas was then passed through a culture-filtrate containing hæmolysin (*Lh* dose 0·3 c.c.). After 2 hours the *Lh* dose was > 1·0 c.c. The hæmolysin had been destroyed.

The same destruction was observed when hæmolysin in culture-filtrates was shaken in an atmosphere of pure nitrogen in a vaccine-shaker for 2 hours, but not when shaken for the same time in a Warburg apparatus. Unlike a Warburg apparatus, in which the surface of the fluid exposed to the gas undergoes little alteration in size during shaking, a vaccine-shaker is designed to " emulsify " and the gas is distributed throughout the medium as minute bubbles. The surface exposed to the gas was therefore enormously increased.

From the above results, it is clear that hæmolysin produced in the synthetic medium was destroyed by violent agitation with an inert gas (pure nitrogen). The possible explanation of such destruction is considered later.

The Rate of Production of α-hæmolysin in a Chemically-defined Medium.

Fig. 1 shows the curve obtained by plotting the *Lh* dose of α-hæmolysin in duplicate cultures against the time of growth when the hæmolysin was harvested. The curve rises steeply for the first 3 days and after that more gradually to the 6th day. After the 9th day there is a gradual fall.

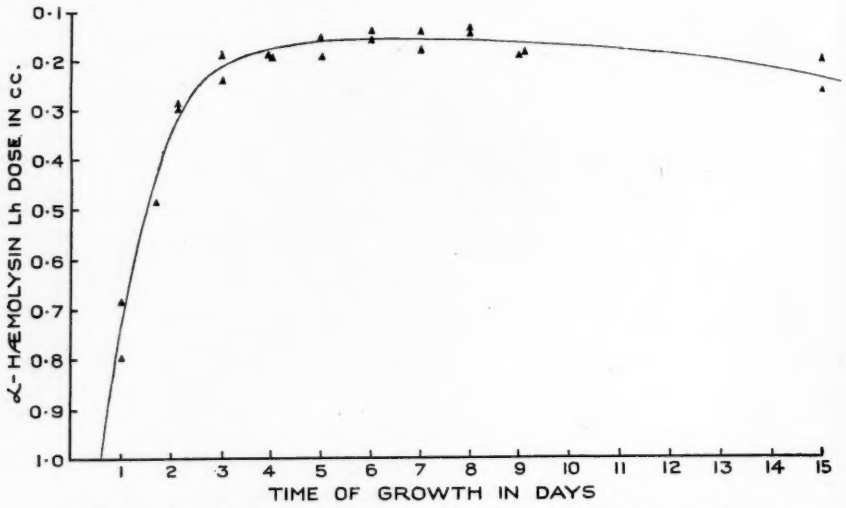

FIG. 1.—The rate of production of *a*-hæmolysin in a chemically-defined medium.

From Fig. 1 a certain variation can be observed in the *Lh* dose. This may be due to slight variations in the oxygen supply, although every effort was made to keep the conditions constant.

Some Properties of Toxin obtained in a Chemically-defined Medium.

Seitz-filtration.

Owing to the absence of " protective colloids " in the medium, *a*-hæmolysin was readily adsorbed when passed through a Seitz filter. A single passage of 15 c.c. through a 6 cm. E.K. filter increased the *Lh* dose from 0·2 c.c. to $>$ 1·0 c.c. The filter-pad, however, could be " saturated " by passing a large quantity (50 c.c.) through it, but even then some increase in the *Lh* dose (from 0·2 to 0·3 or 0·4 c.c.) was apparent.

Dermo-necrotic properties.

Miss M. Llewellyn Smith kindly tested the dermo-necrotic properties of toxin prepared in the chemically-defined medium. She obtained the following results, using a Seitz-filtered sample containing *Lh* dose 0·3 c.c. of *a*-hæmolysin :

The minimum necrotic dose for the skin of a guinea-pig .	0·01 c.c.
Lr/5 dose for the skin of a guinea-pig	0·08 ,,

These results are comparable with those obtained from toxin prepared in the standard laboratory medium.

β-hæmolysin.

That the strain of *Staphylococcus aureus*, Aγ, produced β-hæmolysin was evident from the results obtained by Bigger (1933). It was of interest to discover whether this was produced in the chemically-defined medium. Miss M. Llewellyn Smith investigated this point also. Using monovalent α-antitoxin, she neutralized the α-hæmolysin (*Lh* 0·4 c.c.) in a Seitz-filtered sample and tested the neutralized filtrate with the following results :

β M.H.D. (sheep's red blood-corpuscles)	0·003 c.c.
β *Lh*/10 dose ditto	0·1 c.c.
β M.R.D. (erythema in skin of guinea-pig)	0·0125 c.c.
β *Lr*/10 dose ditto	0·055 c.c.

For the β *Lh* and β *Lr* doses, she used an arbitrary unit of β-antitoxin, equal to 0·01 c.c. of serum 17492 of the National Institute for Medical Research at Hampstead. It corresponded to about 1/9 y unit (Glenny and Stevens, 1935).

Small quantities of β-hæmolysin were thus produced in the chemically-defined medium.

Nitrogen content of culture filtrates containing α-hæmolysin.

Using a Seitz-filtrate of a culture in the amino-acid medium (*Lh* dose of α-hæmolysin 0·3 c.c.), the results given in Table VI were obtained. Figures obtained by McClean (1937), using a cellophane-diffusate of Bigger's veal-peptone-glycerol medium, are included for comparison. It will be seen that, although the total nitrogen of the culture-filtrate from the synthetic medium was nearly a third of that produced in the cellophane-diffusate medium, the amino-nitrogen was much greater.

TABLE VI.—*The Nitrogen Content of Culture-filtrates Containing α-hæmolysin.*

Medium.		Total nitrogen. (mg. per 1 c.c.)	Amino-nitrogen. (mg. per 1 c.c.)
Amino-acid medium optimum for hæmolysin production	Uninoculated . .	0·77	0·662
	Culture-filtrate containing α-hæmolysin (*Lh* 0·3 c.c.)	0·572	0·415
Cellophane-diffusate of Bigger's medium (from McLean, 1937)	Uninoculated . .	1·694	0·412
	Culture-filtrate containing α-hæmolysin	1·442	0·252

Ammonium sulphate precipitation of α-hæmolysin.

Preliminary results showed that α-hæmolysin was readily precipitated by saturated ammonium sulphate solution, as in the case of toxin produced in standard laboratory media. Further work is in progress.

DISCUSSION.

From the above results, it is evident that the composition of the medium is an important factor in hæmolysin production. In particular, the constituent amino-acids play an important part, especially arginine. Gengou (1935), who first observed specific effects with certain amino-acids, attempted to relate the effect of the amino-acids to their molecular structure. He suggested that arginine pre-eminently was required and that other amino-acids were effective in so far as they resembled arginine in chemical structure (*e. g.* ornithine) or could be converted into substances resembling arginine. His work, however, was complicated by the presence of meat-extract in his basal medium. As he himself pointed out, arginine might have been liberated during growth from nitrogenous substances (*e. g.* polypeptides) already present in the medium.

In the present work, in which a chemically-defined medium has been used throughout, only ornithine was to any extent capable of replacing arginine; yet, in the presence of arginine, other amino-acids (notably glycine, proline, phenylalanine and valine) had a definite effect. It might be suggested that each of these amino-acids had a separate use in the synthesis of the hæmolysin molecule. Some hæmolysin, however, was still obtained in the absence of each of these amino-acids and, even without arginine (in the full amino-acid mixture), a small quantity of hæmolysin was observed (Table III). Since the strain of staphylococcus used had been adapted to synthesize all the amino-acids it required for growth, it is likely that it was able to synthesize them in sufficient quantity and to utilize those necessary for producing hæmolysin. The relative importance of the amino-acids might be an indication of the relative difficulty with which they could be synthesized for the purpose by the organism. Even those amino-acids having no apparent effect on hæmolysin production might still be required, but might be synthesized sufficiently easily for hæmolysin production not to be affected by their absence. Arginine is only synthesized with difficulty, even by the adapted strain, and then only in a quantity sufficient for the production of very small quantities of hæmolysin and not sufficient for optimum growth.

Oxygen was another important factor. In order to obtain good and constant yields of hæmolysin, it was not only necessary to have an adequate exposed surface area to a given volume of medium, but also to renew periodically the gas above the culture medium. The effect of oxygen could not be accounted for entirely by its effect on growth, since, for example, a good anaërobic growth produced only traces of hæmolysin.

Of particular interest, in view of the relative stability of staphylococcal hæmolysin, is the effect of passing gases through hæmolysin produced in the synthetic medium. Complete destruction of hæmolysin occurred. This was observed even when an inert gas (nitrogen, free from oxygen) was used. Mechanical shaking with a gas was also effective in destroying hæmolysin. Now all the evidence suggests that hæmolysin is protein in nature (or is associated closely with a protein). If this is so, the destructive effect of gassing and shaking might well be due to denaturation, as is known to occur

with other proteins under similar conditions. It is difficult to reconcile the destruction of hæmolysin produced by gassing with the results obtained by Casman. As previously mentioned, he actually obtained greater yields of hæmolysin when a gas mixture (20 per cent. CO_2 in 80 per cent. O_2) was passed through his cultures (veal-peptone broth). The increased yield was probably due to the more effective oxygenation, but he observed no evidence of destruction. The explanation probably lies in the nature of the medium used. It is well known that inhibitory agents are often less effective in complex media containing proteins or partially hydrolyzed proteins than in more simple media. This may be attributed to a " protective effect " of colloids present in the former.

SUMMARY.

1. Good yields of α-hæmolysin have been obtained in a medium in which all the constituents were chemically known.

2. α-hæmolysin production was sensitive to alterations in the composition of the medium and in the conditions of growth.

3. The effect of various amino-acids, sugars, polyhydric alcohols, organic and inorganic salts was investigated. The most important constituents were the amino-acids : arginine, glycine, proline, phenylalanine and valine.

4. Oxygen and carbon dioxide were important factors in the production of α-hæmolysin in the chemically-defined medium.

5. Indications were obtained of a constituent of acid-hydrolysed edestin stimulating α-hæmolysin production, but not containing a primary amino-group.

6. α-hæmolysin produced in the chemically-defined medium was rapidly destroyed by gassing or shaking with pure nitrogen. It could be precipitated by saturated ammonium sulphate.

7. β-hæmolysin was also present in small quantities.

This work was carried out under the direction of Dr. Paul Fildes to whom I am indebted for much helpful assistance and advice. My thanks are also due to Prof. A. C. Chibnall for providing 500 g. of crystalline edestin, to Dr. G. M. Richardson for active help at an early stage of the work and to Miss M. Llewellyn Smith for generous samples of antitoxin and for carrying out dermo-necrotic tests and tests for β-hæmolysin.

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INFLUENCE OF TRANSMITTED LEUKÆMIA ON METABOLISM OF UNINFILTRATED LYMPHOID TISSUE.

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ALL attempts to transmit mouse lymphatic leukæmia by a cell-free agent have been unsuccessful. Whether leukæmia cells inoculated into animals produce leukæmia in the host by reproduction of the inoculated cells or by stimulation of host-cells is not established with certainly. A cytological approach to this problem was reported by Potter and Richter (1933), who concluded that there was no increase in the production of lymphoid cells by the hosts either in lymphoid tissues or locally from connective tissues. By means of serial sections of all the important regions of series of mice taken at regular intervals after inoculation, the injected cells or their descendants were traced to infiltration and formation of lesions in the host-tissues. These observations indicated that the infiltrating cells of a given line of transmissible lymphatic leukæmia of mice are the descendants of the cells of the original spontaneous case. The same conclusion was reached by Furth (1933).

Another approach to this problem was suggested by the following observations: The metabolism of lymph-nodes of spontaneous (Victor and Potter, 1935*b*) and transmission line (Victor and Wintersteiner, 1934) leukæmia differs from normal. Pre-leukæmic lymph-nodes have metabolic alterations in the direction of malignancy and can transmit leukæmia (Victor and Potter, 1935*a* and 1936). If inoculated leukæmia cells stimulated the host lymphoid tissues to produce the characteristic cells of a transmission line then uninfiltrated lymphoid tissue should have pre-leukæmic metabolic characteristics. Since individual transmission lines have specific metabolic (Victor and Wintersteiner, 1934) as well as clinical, pathological (Richter and MacDowell, 1930) and cytological (Potter and Richter, 1932) characteristics, the quantitative metabolic change in the pre-leukæmic tissue might be controlled by the use of transmission lines with different metabolic rates.

This paper is concerned with the effects of intraperitoneal inoculation of leukæmia cells on the metabolism of uninfiltrated lymphoid tissue of the host.

MATERIAL.

The mice were of strain C58 and 6–8 weeks old. One hundred per cent. were susceptible to various transmission lines of lymphatic leukæmia originating in this strain. Two transmission lines, A and I, were employed; the characteristics of these have been previously reported (Richter and MacDowell, 1930; Victor and Wintersteiner, 1934). During the present experiments the interval between death and inoculation with line I was 3 to 4½ days, with line A 6–8 days. The Q_{O_2}, $Q_{CO_2}^{O_2}$, and $Q_{CO_2}^{N_2}$ of the combined infiltrated lymph-nodes after inoculation with line I when last reported (February to April, 1933) were $5 \cdot 46 \pm 0 \cdot 08$, $7 \cdot 78 \pm 0 \cdot 26$ and $18 \cdot 0 \pm 0 \cdot 36$ respectively (Victor and Potter, 1934), and for line A (October, 1932, to January, 1933) were $6 \cdot 4 \pm 0 \cdot 11$, $1 \cdot 88 \pm 0 \cdot 1$ and $8 \cdot 31 \pm 0 \cdot 33$ respectively (Victor and Wintersteiner, 1934).

METHOD.

Lymph-node biopsies before, and at daily intervals after, inoculation with a transmission line permitted one to follow the metabolism of the lymphoid tissue in the same animal. The day before the first operative procedure the cervical, axillary or inguinal hairs were removed with a barium sulphide paste. The tissues were excised aseptically during ether anæsthesia. All biopsies were performed on peripheral nodes with the exception of those carried out the day the animal was sacrificed. Then the mesenteric node was removed under the same conditions, and at the same time as the peripheral node. Either immediately after or the day following the first biopsy, the mice were inoculated intraperitoneally with the standard dose of the transmission lines. At subsequent daily intervals lymph-node biopsies were performed.

The respiration was observed in Ringer-phosphate solution, pH 7·4, containing 0·2 per cent. glucose and 0·013 *M* phosphate. The gas mixture was pure O_2. Carbon dioxide was absorbed by alkali on filter paper. Aërobic glycolysis was measured by Warburg's (1930) method, in which the tissue was placed in Ringer solution containing 0·2 per cent. glucose and 0·025 *M* $NaHCO_3$ equilibrated with 5 per cent. CO_2 and 95 per cent. O_2. A control respirometer contained tissue in the same solutions and gas mixtures, except that glucose was omitted. Anaërobic glycolysis was measured in the same glucose-containing solution employed for aërobic glycolysis except that the gas mixture was 95 per cent. N_2 – 5 per cent. CO_2. This gas mixture was run through a combustion furnace containing copper filings at 700° C. The amount of tissue employed in each respirometer, of a type previously described (Victor, 1935), varied from 0·5 to 1·5 mg. moist weight.

In previous experiments an error was made in calculating the anaërobic glycolytic rate due to the addition of the aërobic control to the anërobic deflection. During the present experiments this error has been corrected. Recalculation of the previous data on normal peripheral nodes shows no significant

difference between the previous observations and those observed in the present experiments in Table III.

After the metabolic observations the tissues were fixed in Flemming's solution for histological study.

RESULTS.

The individual data are recorded in Tables I and II for lines I and A respectively. The tables list the respiratory and aërobic and anaërobic glycolytic rates of the specific lymph-nodes as well as the absence or approximate degree of infiltration. A summary of the mean metabolic rates of peripheral or mesenteric node and their probable errors and the standard deviations is presented in Table III.

Since previous observations had shown that lymphoid tissue infiltrated with line I or A cells had high glycolytic rates, it was not surprising to find that late in the course of the disease there was a striking increase in the aërobic and anaërobic glycolytic rates of the lymph-nodes of the inoculated mice. This began on the second and fourth day following inoculation of line I and A respectively.

Inhibition of Anaërobic Glycolysis of Uninfiltrated Lymphoid Tissue.

When the data were segregated so that the metabolism of the uninfiltrated lymphoid tissue was analysed, certain unexpected changes were discovered. It appears that a marked depression of the anaërobic glycolytic rate occurs in uninfiltrated lymphoid tissue after intraperitoneal injection of leukæmia cells. This is quite evident from Tables IV and V.

Table IV lists the mean anaërobic glycolytic rates of microscopically uninfiltrated peripheral and mesenteric lymph-nodes of mice inoculated with line I and A cells. Table V shows the differences between the mean anaërobic glycolytic rates of these lymph-nodes before and at daily intervals after inoculation with line I and A. The statistically significant differences are italicized.

The depression of the anaërobic glycolytic rate of uninfiltrated lymph-nodes is manifest sooner after line I, the more virulent leukæmia, than after line A inoculation. In the former there is a 23 per cent. inhibition 1 day after inoculation, while in the latter there is no decrease until 2 days after inoculation, when the decrease is 20 per cent. The decrease becomes greater until it is 42 per cent. on the second day after line I and 35 per cent. on the third day after line A inoculation. The extent of the depression of anaërobic glycolysis of uninfiltrated tissue after the second and third day of lines I and A leukæmia cannot be ascertained from the available data because of the paucity of observations on uninfiltrated tissue after this time.

It is apparent from the above observations that the inhibition is due to the specific effect of specific leukæmia cells. Without further controls the difference between the effects of the 2 lines on the onset of the inhibition acts

TABLE I.—*Metabolic Rates of Individual Lymph-nodes of Mice Before and After Line I Inoculation.*

Mouse No.	Before inoculation.					1 day.					2 days.				
	Q_{O_2}.	$Q^{O_2}_{CO_2}$.	$Q^{N_2}_{CO_2}$.	Lymph-node.	Degree of infiltration.	Q_{O_2}.	$Q^{O_2}_{CO_2}$.	$Q^{N_2}_{CO_2}$.	Lymph-node.	Degree of infiltration.	Q_{O_2}.	$Q^{O_2}_{CO_2}$.	$Q^{N_2}_{CO_2}$.	Lymph-node.	Degree of infiltration.
44233	5·2	1·1	4·2	R.O.A.	—			..			5·7	2·4	2·3	R.I.	+
44234	4·3	3·0	6·8	,,	—			..			4·5	2·4	3·8	,,	++
44454	4·3	2·4	6·7	,,	—			..			5·4	3·1	6·6	,,	++
44455	4·0	3·8	3·4	,,	—			..			3·9	4·9	4·6	,,	+
47538	3·9	2·0	6·3	L.I.	—	5·4	2·2	3·8	R.I.	—	5·3	3·8	3·5	R.I.A.	−
47609	5·7	2·7	5·8	,,	—	4·4	2·7	3·9	,,	—	4·8	1·7	3·6	,,	−
47610	3·0	..	5·4	,,	..	4·1	2·9	6·5	,,	—	3·8	2·3	5·4	,,	+
47540			..			5·3	3·1	4·5	R.I.C.	—	5·6	1·0	6·0	L.I.	+
47573			..			4·5	1·5	4·8	,,	—	4·9	2·8	2·9	,,	+
47574			..			5·7	2·3	4·6	,,	—	5·6	2·2	3·4	,,	+
47826			..					..			5·0	2·0	4·3	L.I.A.	+
47827			..					..			3·3	1·1	2·2	,,	−
47828			..					..			3·1	3·7	3·0	,,	−
47932			..			4·3	1·5	4·1	L.I.	—	4·3	1·1	2·1	L.O.A.	+
47933			..			4·6	2·0	4·3	,,	—	3·9	1·5	2·6	,,	+
47934			..			4·2	2·6	4·0	,,	—	3·3	2·3	2·2	,,	−
48989	5·2	3·2	4·1	R.I.	—	4·5	1·0	4·5	L.I.A.	—	4·2	2·3	2·9	R.I.A.	−
48990	4·5	1·8	5·0	,,	—	4·6	2·2	3·2	,,	—	3·6	4·1	5·7	,,	−
48991	4·1	2·6	4·7	,,	—	4·1	1·4	3·4	,,	—	3·3	1·6	2·5	,,	−
49254	3·9	2·3	4·6	,,	—	3·7	1·7	2·9	R.I.A.	—	3·8	1·7	1·7	R.I.A.	−
49255	4·7	1·3	6·0	,,	—	3·6	1·0	3·6	,,	—	3·7	1·5	2·1	,,	−
											1st day mesenteric node.				
59120	3·5	2·1	3·4	R.I.	—	4·9	1·1	2·2	R.I.A.	—	4·8	2·6	3·5	..	−
49293	4·4	2·0	6·2	,,	—	4·5	3·4	3·5	,,	—	5·3	2·9	4·7	..	−

TABLE I—*continued.*

Mouse No.	3 days.					4 days.					4 days' mesenteric node.			
	Q_{O_2}.	$Q_{\frac{O_2}{CO_2}}$.	$Q_{\frac{N_2}{CO_2}}$.	Lymph-node.	Degree of infiltration.	Q_{O_2}.	$Q_{\frac{O_2}{CO_2}}$.	$Q_{\frac{N_2}{CO_2}}$.	Lymph-node.	Degree of infiltration.	Q_{O_2}.	$Q_{\frac{O_2}{CO_2}}$.	$Q_{\frac{N_2}{CO_2}}$.	Degree of infiltration.
44233	6·8	3·6	6·6	L.I.	+			..					..	
44234	4·5	5·1	7·9	„	+			..					..	
44454	5·5	4·6	10·3	„	++			..					..	
44455	5·7	6·0	8·6	„	+			..					..	
47538	5·8	5·0	9·4	L.O.A.	+			..					..	
47609	3·8	5·3	8·4	„	++	5·8	6·2	10·3	C.	++	4·8	4·2	8·8	++++
47610	3·7	4·6	6·7	„	++			..					..	
47540	5·3	4·1	6·7	R.I.	++	4·7	4·1	9·6	R.I.A.	++++	5·1	5·4	13·0	+++
47573	4·7	3·4	5·7	„	−	5·5	4·7	12·3	„	+++	4·8	3·5	8·4	+++
47574	5·3	5·3	10·4	„	++	4·6	3·9	12·4	„	+++	..	3·5	10·2	+++
47826	3·6	9·8	9·1	L.I.	++	4·6	4·8	6·8	R.I.	+++	5·5	5·3	10·7	++++
47827	4·8	4·9	4·3	„	++	3·8	4·4	5·9	„	++	5·0	4·3	7·9	++++
48728	4·7	3·3	6·8	„	++	4·6	2·0	4·8	„	++	..	2·4	7·1	+++
47932	3·8	5·2	5·3	R.O.A.	+	4·8	5·8	10·8	R.O.C.	+	..	5·0	16·2	++
47933	3·3	3·5	3·0	„	+	4·3	5·0	10·0	„	+++	4·3	..	11·6	+++
47934	3·2	3·6	4·6	„	+			..					..	
48989	4·4	9·2	10·3	L.I.	+			..					..	
48990	3·6	9·3	10·8	„	++			..					..	
48991	5·2	6·2	8·2	„	+			..					..	
	2nd day mesenteric node.													
49254	4·8	5·5	6·9	..	++			..					..	
49255	4·5	6·1	12·7	..	+			..					..	
59120			..					..					..	
49293			..					..					..	

TABLE II.—*Metabolic Rates of Individual Lymph-nodes of Mice Before and After Line A Inoculation.*

Mouse No.	Before inoculation.					1 day.					2 days.					3 days.				
	Q_{O_2}.	$Q^{O_2}_{CO_2}$.	$Q^{N_2}_{CO_2}$.	Lymph-node.	Degree of infiltration.	Q_{O_2}.	$Q^{O_2}_{CO_2}$.	$Q^{N_2}_{CO_2}$.	Lymph-node.	Degree of infiltration.	Q_{O_2}.	$Q^{O_2}_{CO_2}$.	$Q^{N_2}_{CO_2}$.	Lymph-node.	Degree of infiltration.	Q_{O_2}.	$Q^{O_2}_{CO_2}$.	$Q^{N_2}_{CO_2}$.	Lymph-node.	Degree of infiltration.
50187			..			3·9	1·9	5·0	L.O.A.	—	4·7	2·1	2·1	R.I.	—	3·6	2·2	1·9	R.O.L.	—
50188			..			4·7	2·9	4·5	L.I.A.	—	..	1·9	4·2	,,	—	4·3	2·3	2·8	,,	—
50230	4·6	2·3	4·8	L.I.A.	—	3·2	1·8	2·4	R.I.	—	3·9	1·5	5·3	R.I.C.	—	3·9	0·8	3·5	L.I.	—
50231	4·3	2·3	4·2	,,	—	4·2	1·0	4·0	,,	—	4·2	2·0	4·5	R.O.C.	—	3·4	1·4	3·3	,,	—
50291	3·3	2·3	4·1	,,	—	4·6	1·0	6·5	R.I.A.	—	4·1	1·9	4·1	R.O.A.	—	4·1	3·0	3·7	,,	—
50292	3·2	2·8	4·7	,,	—	3·9	2·7	6·2	R.I.C.	—	4·6	2·8	2·6	R.I.A.	—	4·7	1·4	3·5	,,	—
50970	4·3	1·9	2·6	,,	—	4·1	1·4	3·4	L.I.	—	3·9	1·0	2·9	R.I.	—	3·8	2·3	3·5	R.O.A.	—
50971	3·5	1·9	4·7	,,	—	3·5	1·5	3·7	,,	—	3·2	2·1	2·9	,,	—	3·7	1·5	3·0	,,	—
51986	3·2	2·0	4·2	,,	—	4·5	1·6	5·2	R.I.	—	4·2	1·5	3·8	R.O.A.	—	4·7	1·9	3·3	L.I.	—
51987	3·2	3·0	4·5	,,	—	5·0	1·7	5·2	R.O.A.	—	4·1	1·7	4·6	R.I.	—	5·0	2·5	2·6	,,	—
51988	4·1	3·0	5·8	,,	—	4·4	2·1	4·6	R.I.A.	—	4·2	2·4	5·0	,,	—	5·0	1·4	4·1	,,	—
																2 days' mesenteric node.				
50758	4·0	1·1	4·2	,,	—	4·1	1·7	3·5	R.I.	—	4·4	1·5	3·4	L.I.	—	4·3	1·8	4·3	..	—
50759	3·8	2·0	2·9	,,	—	4·3	2·2	3·3	,,	—	3·8	2·3	2·7	,,	—	4·1	1·7	2·7	..	—
52170	..	2·0	5·8	R.O.A.	—	..	1·4	5·4	L.I.	—	..	1·8	3·1	R.I.	—	..	2·2	3·9	..	—
52171	4·5	3·1	6·1	,,	—	..	1·9	5·5	,,	—	..	1·8	4·3	,,	—	5·1	1·0	3·5	..	—
52332	..	1·5	4·9	,,	—	..	1·1	5·9	,,	—	..	1·1	2·7	,,	—	3·6	1·7	3·0	..	—

TABLE II—*continued.*

Mouse No.	4 days.					5 days.					6 days.					6 days' mesenteric node.			
	Q_{O_2}.	$Q^{O_2}_{CO_2}$.	$Q^{N_2}_{CO_2}$.	Lymph-node.	Degree of infiltration.	Q_{O_2}.	$Q^{O_2}_{CO_2}$.	$Q^{N_2}_{CO_2}$.	Lymph-node.	Degree of infiltration.	Q_{O_2}.	$Q^{O_2}_{CO_2}$.	$Q^{N_2}_{CO_2}$.	Lymph-node.	Degree of infiltration.	Q_{O_2}.	$Q^{O_2}_{CO_2}$.	$Q^{N_2}_{CO_2}$.	Degree of infiltration.
50187	4·4	2·0	4·8	L.I.	—	4·7	4·8	10·2	R.I.A.	+	3·4	4·5	7·6	L.O.C.	+	4·6	2·6	10·4	+++
50188	4·3	2·8	5·2	,,	—	4·3	4·6	8·7	,,	+	4·9	4·1	6·9	,,	+	4·4	4·0	14·6	+++
50230	3·9	2·1	3·6	R.I.A.	—	3·0	2·0	2·2	L.O.A.	—	3·6	2·4	4·9	R.O.A.	+	3·1	5·0	14·8	+++
50231	4·0	2·1	3·5	,,	—	3·0	2·1	4·2	,,	—	3·6	3·0	6·8	,,	+	..	2·9	8·8	+++
50291	4·4	3·3	5·6	R.I.	+	4·3	2·9	10·7	R.I.C.	+	4·1	1·5	4·1	R.I.A.	—	4·3	2·3	7·9	++
50292	3·8	2·1	8·1	L.I.C.	+	4·0	3·6	13·2	R.I.	++	4·3	2·8	4·0	,,	—	4·0	3·6	12·1	++++
	3 *days' mesenteric node.*																		
50970	3·7	3·5	7·2	..	+			..					..					..	
50971	4·3	2·7	3·4	..	—			..					..					..	
51986	3·0	1·6	6·0	..	+			..					..					..	
51987	4·1	2·1	3·1	..	—			..					..					..	
51988	4·3	2·6	3·6	..	—			..					..					..	
50758			..					..					..					..	
50759			..					..					..					..	
52170			..					..					..					..	
52171			..					..					..					..	
52332			..					..					..					..	

TABLE III.—*Metabolism of Peripheral and Mesenteric Lymph-nodes Before and After Leukæmia Inoculation.*

Number of obs.	Days after inoculation.	Lymph-node.	Q_{O_2}. Mean.	Q_{O_2}. SD.	$Q^{O_2}_{CO_2}$. Mean.	$Q^{O_2}_{CO_2}$. SD.	$Q^{N_2}_{CO_2}$. Mean.	$Q^{N_2}_{CO_2}$. SD.
			Line I.					
24	Prev. data	Peripheral	4·60± ·08	·52	1·92± ·09	·69	4·88± ·17	1·13
14	Before	,,	4·34± ·12	·66	2·33± ·12	·67	5·18± ·21	1·15
16	1	,,	4·53± ·09	·52	2·04± ·12	·74	3·99± ·15	·92
21	2	,,	4·33± ·13	·86	2·13± ·13	·82	3·55± ·22	1·49
19	3	,,	4·62± ·14	·94	5·37± ·30	1·93	7·53± ·34	2·21
9	4	,,	4·74± ·13	·59	4·54± ·26	1·16	9·21± ·58	2·60
9	4	Mesenteric	4·92± ·09	·32	4·45± ·19	·39	10·43± ·19	·86
			Line A.					
14	Before	Peripheral	3·84± ·09	·46	2·23± ·10	·56	4·54± ·17.	·94
16	1	,,	4·18± ·10	·51	1·74± ·09	·55	4·64± ·19.	1·15
16	2	,,	4·11± ·07	·36	1·84± ·07	·44	3·64± ·15.	·92
5	2	Mesenteric	4·27± ·22	·58	1·68± ·13	·39	3·48± ·17.	·58
11	3	Peripheral	4·20± ·11	·55	1·88± ·13	·62	3·20± ·14.	·57
5	3	Mesenteric	3·88± ·17	·49	2·5 ± ·21	·64	4·70± ·49.	1·62
6	4	Peripheral	4·13± ·09	·30	2·4 ± ·14	·48	5·15± ·40.	1·48
6	5	,,	3·88± ·20	·68	3·33± ·34	1·12	8·20± 1·25.	4·56
6	6	,,	3·98± ·16	·54	3·05± ·30	1·01	5·72± ·39.	1·42
6	6	Mesenteric	4·08± ·18	·53	3·40± ·28	·92	11·43± ·73.	2·67

TABLE IV.—*Anaërobic Glycolytic Rates of Uninfiltrated Lymph-nodes Before and After Line I and A Inoculation.*

Day after inoculation.	Number of obs.	$Q^{N_2}_{CO_2}$. Line I. Mean.	$Q^{N_2}_{CO_2}$. Line I. SD.	Number of obs.	$Q^{N_2}_{CO_2}$. Line A. Mean.	$Q^{N_2}_{CO_2}$. Line A. SD.
Before	14	5·18 ± ·21	1·15	14	4·54 ± ·17	·94
1	16	3·99 ± ·15	·92	16	4·64 ± ·19	1·15
2	10	2·94 ± ·24	1·19	21	3·60 ± ·13	·87
3		..		14	3·21 ± ·08	·44

TABLE V.—*Mean* $Q^{N_2}_{CO_2}$ *Differences between Uninfiltrated Lymph-nodes Before and After Line I and A Inoculation.*

Comparison of	Line I. Difference.	Line I. Difference. / P.E. difference.	Line A. Difference.	Line A. Difference. / P.E. difference.
Before and 1	*1·19 ± ·26*	4·2	·10 ± ·25	·4
Before and 2	*2·24 ± ·32*	7·0	*·94 ± ·22*	4·3
1 and 2	*1·05 ± ·28*	3·7	*1·04 ± ·23*	4·5
Before and 3	..	..	*1·33 ± ·19*	7·0
1 and 3	..	..	*1·43 ± ·21*	7·0
2 and 3	..	..	·39 ± ·15	2·6

Italicized differences are statistically significant.

as a control on the effect of lymphoid tissue inoculation and biopsy procedure. However, further controls were obtained with the following procedures :

(1) Daily lymph-node biopsies were performed on 4 normal mice for 5 consecutive days.

(2) The biopsy procedure was carried out 1 and 2 days before the leukæmia cell inoculation.

(3) Normal spleen in doses equivalent to the leukæmic tissue from mice of strain C58 was injected intraperitoneally in 4 normal mice. Metabolic observations were carried out on biopsy lymph-nodes before and at daily intervals after inoculation.

(4) The same procedure as (3) was carried out with normal spleen from mice of another strain, namely Storrs-Little.

These various control procedures showed that neither the operation nor normal lymphoid nor splenic tissue inoculation produce any inhibitory effect on the anaërobic glycolytic rate of lymphoid tissue. For lack of space these negative data are not presented.

On the Nature of the Inhibition of Anaërobic Glycolysis of Uninfiltrated Lymphoid Tissue of Mice Inoculated with Leukæmia.

The depression occurs in tissue free of leukæmic cells. It does not appear immediately after inoculation of leukæmia cells, for with line A it is not apparent until 2 days later. Both findings indicate that the inhibition of anaërobic glycolysis of uninfiltrated lymphoid tissues of leukæmia hosts is not due to the direct action of leukæmia cells. Additional evidence has been gathered confirming this statement.

If the depression were due to the direct effect of leukæmia cells on normal cells, then a mixture of leukæmia and normal cells should have a lower anaërobic glycolytic rate than the rate calculated from the metabolism of either. The results of experiments designed to test this point are recorded in Table VI.

TABLE VI.—*Mean Anaërobic Glycolytic Rates of Mixtures of Normal and Line I or Line A Lymphoid Cells (Comparison with their Separate Glycolytic Rates).*

	Number of obs.	Leukæmia. Weight. (mg.)	Leukæmia. $Q_{CO_2}^{N_2}$.	Normal. Weight. (mg.)	Normal. $Q_{CO_2}^{N_2}$.	Mixture observed $Q_{CO_2}^{N_2}$.	Mixture calculated $Q_{CO_2}^{N_2}$. From separate rates.	Difference (%).
Line I	15	38·6	14·10	52·8	5·13	9·15	9·10	0·21
Line A	6	46·2	9·57	43·5	4·65	7·20	7·20	0·25

There were 15 sets of observations with line I and 6 with line A. The procedure employed was to measure the metabolism of normal and leukæmia cells in

separate respirometers of the Fenn (1927) type. At the same time the metabolism of a mixture of the two tissues derived from the same normal and leukæmic hosts was studied. In some of the experiments the total metabolism was observed with the two types of tissue in separate parts of the same respirometer, and subsequently the tissues were mixed in the same chamber. The experiments showed no significant differences between the separate and mixed anaërobic glycolytic rates of normal and leukæmia lymphoid cells.

However, there remains the remote possibility that, in the presence of leukæmia cells, normal cells lose glycolytic material or systems which may be taken up by the leukæmia cells. In other words, in a mixture of normal and leukæmia cells, a depression of the glycolytic rate of normal tissue might be obscured by increases in the glycolytic activity of leukæmia cells.

If leukæmia cells secreted substances that directly depressed the anaërobic glycolysis of normal lymphoid cells, such depressors should be demonstrable in fluids exposed to leukæmia cells. This was tested by exposing 60–80 mg. of line I or A cells for 1 hour in 1 c.c. of Ringer solution containing 0·025 *M* Na HCO_3 and 0·4 per cent. glucose (optimum concentration for maximum anaërobic glycolysis) and 5 per cent. CO_2 – 95 per cent. O_2 gas mixture. Then the cells were removed by centrifugation and the solution tested, under anaërobic conditions in 95 per cent. N_2 – 5 per cent. CO_2, on lymphoid tissue of normal mice by comparison with fresh Ringer-bicarbonate-glucose solution of similar composition. For the tests about 1 mg. of normal lymphoid tissue from the same node was placed in 40 cm. of the fluid in each of two respirometers of the type used in the earlier part of this investigation. The results of 7 sets of observations with fluid exposed to line A cells gave a mean $Q\,^{N_2}_{CO_2}$ of 5·4 with a control mean of 5·8, 4 sets with line I yielded means of 5·8 and 5·4 for the experimental and control fluids respectively. No evidence was obtained for the presence of an inhibiting substance in fluids exposed to leukæmia cells.

DISCUSSION.

From this investigation it is apparent that the pre-leukemic rise in $Q\,^{N_2}_{CO_2}$ preceding spontaneous lymphatic leukæmia does appear in mice inoculated with transmissible leukæmia. The present physiological observations confirm the morphological findings of Potter and Richter (1933) and of Furth *et al.* (1933). These indicate that after leukæmia cell injection the host lymphoid tissues do not elaborate leukæmia cells, but that the infiltrating cells of a given line of transmissible lymphatic leukæmia are the descendants of the original spontaneous case.

That leukæmia cells injected into susceptible mice initiate a chain of events leading to depression of the anaërobic glycolytic rates of uninfiltrated lymphoid tissues is obvious from these experiments. This response is not only specific for leukæmic tissue but is also specific for the individual transmission line. The effect is not observed with splenic and lymphoid tissue injection of normal mice of the same or foreign strains. With transmission line I inhibition is apparent 1 day after injection while line A requires 2 days for the same effect.

The mechanism of this inhibited glycolytic activity of uninfiltrated lymphoid tissue is not clear. The degree of inhibition is influenced by time and/or by the quantity of leukæmic tissue in the host, since the amount of leukæmic tissue increases with the interval after inoculation. The inhibition occurs in tissue free from leukæmia cells as well as in tissue containing leukæmia cells (Tables I and II). Therefore the "inhibitor" must be separate from the leukæmia cells and must circulate in tissue fluids. *In vitro* mixing of leukæmic and normal cells in Ringer solution or exposing normal cells to Ringer solution that had previously bathed leukæmia cells, did not demonstrate any inhibitor that could be separated from the leukæmia cells. It has recently been found that the anaërobic glycolytic rate of line I cells in serum from mice with line I leukæmia is lower than in normal mouse-serum (Victor and Potter, 1938). Whether leukæmia serum has a similar effect on normal cells has not been investigated, but that it does is strongly suggested from the present experiments.

Profound physiological changes occur in transmissible lymphatic leukæmia of mice. Some of these have been discussed elsewhere (Victor and Potter, 1938). It will be desirable to ascertain whether this inhibition occurs in other tissues. The question as to whether the inhibition of the anaërobic glycolytic activity of uninfiltrated tissues is a fundamental characteristic of the cachexia that marks this disease in man as well as in mice, awaits the results of future investigation for an answer.

SUMMARY AND CONCLUSION.

The intraperitoneal inoculation of a saline suspension of leukæmic spleen or lymph-nodes of two different transmission lines depressed the anaërobic glycolytic rates, but not the respiratory nor aërobic glycolytic rates of uninfiltrated lymphoid tissue. The inoculation of similar amounts of normal spleens of two different strains of mice produced no such effect. No inhibition of anaërobic glycolysis could be demonstrated when normal and leukæmia lymphoid cells were mixed in Ringer solution, nor when normal cells were suspended in Ringer solution that had previously bathed leukæmia cells.

The inhibition of the anaërobic glycolytic activity of uninfiltrated lymphoid tissue of mice with transmitted lymphatic leukæmia depends on the following factors:

(1) Specific response to individual transmission lines.

(2) Interval after inoculation and/or quantity of leukæmic tissue in the host.

(3) A humoral factor which is either a product of leukæmia cells or the resultant of a reaction between host and leukæmia cells.

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